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EPIGENETIC REGULATION OF A GENE, *MS-1*, IN CELLS OF DIFFERENT METASTATIC POTENTIAL

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By

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ABSTRACT

Breast cancer is the most common malignancy and a major cause of cancer-related death among Canadian women. Although treatment of primary breast tumours is highly successful through surgery, metastatic breast cancer is difficult to treat. Cancer progression and metastasis require the accumulation of numerous genetic and epigenetic alterations. Normal cells that acquire such alterations can transform into cancer cells, resulting in primary tumour formation. Primary tumours are a heterogeneous population, containing cells of various metastatic potentials. Cells that acquire a high potential for metastasis can spread to secondary locations. Our model system consists of two subpopulations, with different metastatic potential, derived from the same rat mammary adenocarcinoma. Using this model, a differentially expressed novel gene, termed MS-1, was discovered. Due to significant expression of this gene in the poorly metastatic subpopulation and lack of expression in the highly metastatic subpopulation, MS-1 may have involvement in metastasis suppression. Several breast cancer metastasis suppressor genes have been identified on the basis that they are down-regulated during the progression of metastasis. Epigenetic mechanisms, such as DNA methylation, account for loss of expression in several of these genes. Hypermethylation of CpG islands within gene promoters results in deacetylation of histone proteins and produces a compact chromatin structure that is unfavourable for transcription. A CpG island spans the 5' untranslated region, exon 1 and part of intron 1 of the MS-1 gene. Our data reveal

aberrant methylation patterns of this CpG island between the cell lines of different metastatic potential in our model. Also, MS-1 expression was partially induced by both DNA methylation and histone deacetylation inhibitors. Following a screen of several cancer cell lines of varying metastatic potential, it appears that the presence of DNA methylation in the CpG island of MS-1 correlates with the lack of MS-1 expression. Therefore, these results suggest that MS-1 may be silenced in cells of high metastatic potential through epigenetic mechanisms.

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Several changes have occurred in my life throughout the duration of this research endeavor. My mother Merrilee and her courageous struggle with cancer gave me the strength to fight my own battles and the realization of what truly matters. The new-found love between my father Jerald and step-mother Shelley inspired me to believe that your dreams will come true if you face life with open arms. I also dedicate this work to my long-time friend Danielle, who is the sister I never had, and my brother Jeremy, who is so much more than that.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
α-MEM	Alpha-minimal essential medium
APL	Acute promyelocytic leukemia
ATCC	American Type Culture Collection
ATF	Activating transcription factor
BCS	Bovine calf serum
BRCA1	Breast cancer susceptibility protein 1
BRMS1	Breast cancer metastasis suppressor 1
bZIP	Basic leucine zipper
CAb	Complement antibody
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	Complementary deoxyribonucleic acid
CGBP	Unmethylated-CpG-binding protein
COBRA	Combined bisulfite restriction analysis
CpG	Cytosine-phosphate-Guanine
CRE	Cyclic AMP-response element
CREB	cAMP-response element binding protein
CREB3L1	cAMP-response element binding protein 3-like 1
DAC	5-aza-2'-deoxycytidine
DAPK	Death-associated protein kinase
dATP	Deoxyadenosine triphophate
dCTP	Deoxycytidine triphosphate

DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagles medium
DMH	Differential methylation hybridization
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
ds	Double stranded
DTT	Dithiothreitol
ECIST	Expressed CpG island sequence tag
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERSE	ER stress-response element
EST	Expressed sequence tag
FBS	Fetal bovine serum
GAPD	Glyceraldehyde-3-phosphate dehydrogenase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
hMS-1	Human MS-1
HMT	Histone methyltransferase
HP1	Heterochromatin protein 1
iGb_4Cer	Isoglobotetraosylceramide, isogloboside
KAI1	Kangai protein1
LB	Luria-Bertani
LG_3	Lung three

LN_4	Lymph node four
LOH	Loss of heterozygosity
MBD	Methyl-CpG-binding domain protein
MDM2	Mouse double minute
MeCP2	Methyl-CpG-binding protein
MGMT	O6-methylguanine-DNA methyltransferase
MKK4	Mitogen-activated protein kinase 4
MOPS	3-[N-mopholino] propane sulfonic acid
mRNA	Messenger ribonucleic acid
MSP	Methylation-specific PCR
MTA	5'-methylthioadenosine
MTA1	Metastasis-associated protein 1
MTHFR	Methylene-tetrahydrofolate reductase
NCBI	National Center for Biotechnology Information
NCoR	Nuclear hormone receptor corepressor
NuRD	Nucleosome remodeling histone deacetylase
OASIS	Old Astrocyte Specifically Induced Substance
ORF	Open reading frame
PBI	Plant Biotechnology Institute
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PML	Promyelocytic leukemia protein
RAR	Retinoic acid receptor
Rb	Retinoblastoma protein
RBP1	Rb-binding protein 1

RIP	Regulated intramembrane proteolysis
rMS-1	Rat MS-1
RNA	Ribonucleic acid
RNase	Ribonuclease
RP58	Repressor protein 58
RT	Room temperature
S1P	Site-1 protease
S2P	Site-2 protease
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
ss	Single stranded
SSC	Standard saline citrate
TAE	Tris-Acetic acid-EDTA
TE	Tris-EDTA
TSA	Trichostatin A
uPA	Urokinase plasminogen activator
UPR	Unfolded protein response
UV	Ultraviolet
XBP1	X-box binding protein 1
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1.0 LITERATURE REVIEW

1.1 Breast Cancer and Metastasis

Breast cancer is the most common malignancy and a major cause of cancer-related death among Canadian women. One in nine women is expected to develop breast cancer during her lifetime and one in 27 will die of it [1]. Most women succumb to breast cancer if tumours metastasize but cure rates exceed 90% if breast carcinomas remain confined to breast tissue [2]. However, 5-year survival rates fall below 20% if secondary metastases form [3]. Statistics show that early detection is essential for optimal success in the treatment of breast cancer. However, a better understanding of the metastatic process in human breast cancer should translate into substantial improvements in therapeutic outcome for breast cancer patients [2].

Further understanding could lead to the identification of markers on cells with a high probability of causing macroscopic metastases. Genetic markers could be used for diagnosis of tumour type, grade and maybe disease stage, including prediction of metastases location. Also, a better understanding of metastasis could result in identifying gene targets for gene therapy, which may be used as an alternative to chemotherapy [4]. The identification of genes involved in metastasis could be exploited to alter the metastatic cascade. For example, a mimetic could be developed either to prevent the

establishment of new metastases or to block the growth of metastases and possibly induce regression of the metastases [3].

The characteristics of transformed cells include being anchorage-independent, contact-uninhibited, immortal and having a several-fold decrease of genomic stability compared to normal cells. Genomic instability appears to be the driving force for the accumulation of genetic defects required for cells to become fully tumourigenic [2]. Tumour progression is the evolution of already tumourigenic cells towards increasing malignancy. Malignancy is characterized by pathologists on the bases of morphologic attributes, including less differentiated cytology, vascularity, necrosis, mitotic index, and aneuploidy. Hallmarks of malignancy include invasion of cells through the basement membrane and/or metastasis [3].

Metastasis is the progressive growth of cells at a site that is discontinuous from the primary tumour [3]. Malignant cells invade adjacent tissues and penetrate the lymphatic and/or circulatory systems. Cells can travel independently or as emboli that are composed of tumour cells only (homotypic) or of tumour cells and normal cells (heterotypic). Cells or emboli arrest at a secondary site either due to a physical limitation or to binding specific molecules in particular organs or tissues. Tumour cells then proliferate in the vasculature or extravasate into surrounding tissue [2]. Cells are able to spread through the blood vasculature, lymphatics or within body cavities. Primary tumours contain a heterogeneous population of cells with various metastatic potentials [3]. Metastatic potential increases with the acquired the ability to complete each step of the metastatic cascade. Low metastatic potential may be due to inherent deficiencies in

the tumour cells themselves caused by genetic alterations or to defective responses to the host environment as a result of epigenetic regulation [3].

1.2 Epigenetics

1.2.1 Epigenetic Code

Epigenetics is the inheritance of information based on gene expression levels, whereas genetics is the transmission of information based on gene sequences [5]. Epigenetic codes are potentially heritable but can be modified [6]. An epigenetic trait is inherited during DNA replication and cell division but is independent of the DNA nucleotide sequence itself [7] [8] [9]. Variations in DNA methylation and histone modifications constitute a distinct epigenetic code that regulates gene expression. For example, methylated DNA, deacetylated histones, some methylated histone forms and condensed chromatin are associated with inaccessible DNA and repressed or silenced gene expression whereas unmethylated DNA, some acetylated histone forms and open chromatin are associated with active or potential gene expression [6].

1.2.2 Chromatin Structure

Chromatin is the complex of DNA and proteins in which the genetic material is packaged inside the nucleus. It is organized into condensed heterochromatin and open euchromatin. The nucleosome core particle includes ~145 bp of DNA wrapped around a histone octamer core [10] [11]. Histones are the basic protein components of chromatin. The nucleosome core particle consists of two copies each of histones H2A, H2B, H3 and

H4 [7] [11]. The basic unit of DNA structure is the nucleosome. Nucleosomes are connected by ~10-80 bp of DNA, and are compacted by the linker histone H1 into chromatin fibres of ~30 nm in diameter [6]. The complex of the histone octamer, linker histone, and 166 bp of nucleosomal DNA is termed the chromatosome [11]. The DNA wound around the histone octamer is accessible to regulatory proteins. The histone tails are also accessible, and enzymes can chemically modify them to promote nucleosome movement and unwinding [7].

There is considerable evidence to suggest that histones are not merely packaging factors, but function to regulate gene expression. Histones facilitate gene activation by promoting specific structural interactions between distal sequences and facilitate repression by blocking the binding sites for transcription activators. The rate-limiting biochemical response that leads to activation of gene expression involves alterations in chromatin structure. The most compact form is inaccessible and provides a poor template for biochemical reactions. There are several ways the chromatin structure can be altered: the histones of the nucleosome can be covalently modified or histone variants can replace the core histones [7].

1.2.3 CpG Islands

DNA methylation occurs at carbon 5 of cytosine residues 5' to guanine residues, or CpG dinucleotides. The CpG dinucleotide has been progressively depleted from the eukaryotic genome during evolution due to spontaneous deamination of the unstable base 5-methylcytosine to uracil [12]. Methylated cytosine accounts for ~1% of the total DNA bases; however, ~70% of all CpG dinucleotides are methylated [13]. Regions in the

genome that contain a high frequency of CpG dinucleotides are referred to as CpG islands if they meet the following criteria: size ranging from 0.2 to 5 kb, occurring approximately every 100 kb, GC-rich (60-70%) and having a CpG/GC ratio greater than 0.6 [12]. The human genome contains ~29,000 CpG islands [13]. They generally span the 5' end of some genes, including the promoter, untranslated region and exon 1 [14]. Most CpG islands remain unmethylated and are associated with highly active genes, such as housekeeping genes [13] [15].

The transcription of genes is favourable when the appropriate transcription factors are available, the histones are acetylated and unmethylated, and the cytosines in the CpG island, if present, remain unmethylated [14]. CpG islands are generally protected from methylation. Fully methylated CpG islands are found mostly in promoters of retroviruses, transposons, silenced alleles, such as imprinted autosomal genes and genes on the inactivated X-chromosome of females [13]. Normal protection of CpG islands from methylation is mediated by certain transcription factors. For example, binding sites for the transcription factor Sp1 appear to be critical for protection from methylation [8].

1.2.4 DNA Methylation

Genome stability and normal gene expression are maintained by a fixed and predetermined pattern of DNA methylation [14]. DNA methylation protects the genome from invading foreign DNA elements [6]. DNA methylation is mediated by two classes of DNA methyltransferases (DNMTs). The methylated state of the parent DNA strand is maintained in the daughter strands by DNMT1, a maintenance methyltransferase.

DNMT1 recognizes the hemimethylated site and methylates the unmethylated cytosine, restoring the symmetrically methylated CpG dinucleotide pair [16].

DNMT3a and DNMT3b are responsible for *de novo* methylation. DNMT3a and DNMT3b do not have a preference for hemimethylated CpG sites and can methylate unmodified CpG sites as well [6]. DNMT3a and DNMT3b are structurally similar to DNMT1 but with a regulatory region attached to the catalytic domain. The regulatory region binds a variety of transcriptional repressors [17]. *De novo* methylation usually occurs outside of a promoter CpG island and progressively spreads to the center of the island. There are sequences in the promoter region that serve as a docking site for repression complexes including histone deacetylases (HDACs), methyl-CpG-binding proteins (MBDs), DNMTs and other proteins [18].

DNA methylation involves the addition of a methyl group to the DNA. A cysteine SH group from the active site of the DNMT initiates nucleophilic attack at the C6 position of the target cytosine by transient protonation of the cytosine ring at the endocyclic nitrogen, N3, creating cytosine-4,5-enamine. This structure attacks the sulphonium linked methyl group of S-adenosylmethionine (SAM). Following methyl transfer, a proton is abstracted from C5, allowing reformation of the 5,6 double bond [9]. 5-aza-2'-deoxycytidine (DAC) is a cytosine analogue that sequesters DNMT after its incorporation into genomic DNA and therefore, functions as a DNMT inhibitor [18].

DNMTs methylate a specific target cytosine within a DNA molecule. To achieve this specificity the enzyme needs to bring the target cytosine to its catalytic pocket. The target cytosine is rotated on its sugar-phosphate backbone so that the target base is

flipped out into a typically concave active site pocket. This base-flipping phenomenon is observed in several DNA methyltransferases [9].

Other mechanisms for DNMT specificity exist. Eukaryotic DNMTs exhibit no sequence specificity other than the CpG dinucleotide. Other factors are likely required to mediate the regional genomic specificity they exhibit. Targeting of DNMTs to particular genomic regions may occur through protein-protein interactions. For example, DNMT1 is able to bind Rb, the retinoblastoma tumour suppressor protein, which is targeted to a specific set of genes through interaction with the sequence-specific DNA-binding factor E2F [17]. Methyl-CpG-binding protein, MeCP2, forms complexes with hemimethylated as well as fully methylated DNA. DNMT1 associates with MeCP2 in order to perform maintenance methylation [9]. DNMT3a binds RP58, a DNA-binding transcriptional repressor, leading to methylation independent repression of the gene at an RP58-responsive promoter [17]. DNMT3a also associates with HDAC1, leading to histone deacetylase-mediated gene silencing [9]. DNMT3a co-localizes with HP1, a heterochromatin protein, which binds to methylated histones. This association could be important in directing DNA methylation to chromatin that contains methylated histones, leading to long-term silencing [17].

Since DNA can be methylated, it is not surprising that it can also be demethylated. Demethylation occurs during DNA replication. Mechanisms include the loss of fidelity in DNMT1 maintenance methylation, exclusion of DNMT1 from replication complexes and limiting DNMT1 levels. The result is that DNA methylation patterns are not accurately copied to daughter strand DNA, generating hemimethylated CpG sites that can become fully demethylated after rounds of DNA synthesis. The lack

of methylation in CpG islands appears to be passive, either through protection from methylation machinery or by the displacement of such machinery. For example, transcription factor Sp1 can bind to a methylated site and sterically exclude methyltransferases [6]. It has been shown that SAM actively inhibits demethylase activity and can inhibit expression of urokinase plasminogen activator (*uPA*), a metastasis-promoting gene, through induction of methylation-dependent silencing [19]. The balance between methylation and demethylation is proposed as a mechanism for switching the transcription of genes on or off [9].

1.2.5 Histone Modification

Tails of histone amino acids protrude from the nucleosome and are sites of post-translational modification such as acetylation, methylation, phosphorylation, sumoylation and ubiquitination [20]. Possible histone modifications represent a complex set of epigenetic information with combinatorial potential known as the histone code. Histone interactions with DNA and other proteins are affected by these modifications. Chemical modifications, whether on the histone tails extending from the nucleosome surface or within the body of the octamer, serve as signals for the binding of specific proteins [6].

The N-terminal tails on core histones are substrates for reversible lysine acetylation [21]. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) govern the acetylation state of histones. HDACs remove the acetyl group from histones using a charge-relay mechanism consisting of two adjacent histidine residues, two aspartate residues and one tyrosine residue, and crucial for this charge-relay system is a

Zn^{2+} ion, which binds deep in the pocket of the enzyme. Inhibitors such as Trichostatin A (TSA), function by displacing the zinc atom [10].

There are 19 deacetylase enzymes in humans, divided into three classes based on differences in structure and function. The functions of these enzymes include: controlling gene expression through targeted and non-targeted chromatin deacetylation, promotion of transcriptional repression, and deacetylation of other non-histone proteins including transcription factors [21]. Transcriptional activators are often associated with HATs, and repressors often interact with HDACs [22].

The most studied group of HDACs are those of mammalian class I including HDAC1, HDAC2, HDAC3 and HDAC8. HDAC1/2/3-containing complexes serve as corepressors for many chromatin and transcriptional regulators. For example, these HDACs collaborate with ATP-dependent chromatin remodelers. The HDAC1/2 complex associates with DNMTs, which are also recruited by transcriptional regulators to repress transcription, and histone methyltransferases (HMTs). HDAC1 interacts with topoisomerase II, an enzyme that is essential for chromosome condensation and may be involved in gene silencing [21].

There are three main mechanisms of histone deacetylase regulation: subcellular compartmentalization, post-translational modification and formation of multisubunit deacetylase complexes [21]. Association of certain proteins lead to cytoplasmic localization of deacetylases by enhancing nuclear export whereas binding of other chaperones stimulates nuclear localization. Increased phosphorylation of HDAC1/2 disrupts their complexes but specific phosphorylation stabilizes their complexes and stimulates deacetylase activity. Sumoylation of HDAC1 is required for inducing cell-

cycle arrest and apoptotic responses. HDACs and their components are also targeted for proteolytic processing by ubiquitin-dependent degradation. Interaction of HDACs with certain transcriptional factors dictates targeting specificity to distinct genes or chromatin domains [21]. For example, DNA-binding factors recruit the Sin3-HDAC complex and result in histone deacetylation, leading to gene silencing [22].

The Sin3 complex is comprised of at least seven subunits, including HDAC1/2 and Sin3. There is an extensive list of mammalian proteins that repress transcription through use of the Sin3 complex [22]. Nuclear hormone receptor binds to specific promoters in the absence of hormone to repress transcription. Hormone binding produces a conformational change in the complex, converting it to a transcriptional activator with help from other recruited proteins. Unliganded retinoic acid receptor and thyroid hormone receptor interact with corepressor proteins SMRT (silencing mediator of retinoid and thyroid hormone receptor) and NCoR (nuclear hormone receptor corepressor). SMRT and NCoR lead to transcriptional repression by recruiting the Sin3-HDAC complex. Also, the methyl-CpG-binding protein MeCP2 is able to recruit the Sin3-HDAC complex to methylated DNA.

The mammalian NuRD (nucleosome remodelling histone deacetylase) complex also has seven subunits including: HDAC1/2, MTA2 and MBD3. MTA2 is related to MTA1 (metastasis-associated protein 1), which is highly expressed in metastatic cells. MBD3 (methyl-CpG-binding-domain protein 3) involvement suggests that nucleosome remodelling and histone deacetylation abilities of the NuRD complex are targeted to methylated regions of the genome [22].

There are several possible mechanisms for repression by histone deacetylation. Histone deacetylation may specifically prevent the assembly of the transcription initiation complex [22]. Localized histone deacetylation may stabilize nucleosomal structure and internucleosomal histone-histone interactions to inhibit the DNA accessibility of transcriptional activators and transcriptional machinery [21]. Normally, there is a strong association between negatively-charged DNA and the positively-charged histones. Acetylation neutralizes the charge on lysine residues within the histone tail, weakening the DNA-histone association and allowing the binding of transcription machinery. Deacetylation restabilizes this interaction. Acetyl-lysine residues may serve as a signal for transcriptional regulators and removal of this signal by deacetylation might impair the assembly or recruitment of transcriptional activators [22].

Deacetylation specific factors can inhibit RNA Polymerase I-dependent transcription, suggesting that transcription machinery may be directly targeted [21]. Acetylation of sequence-specific transcriptional activators has been found to alter their DNA-binding ability, activation potential, stability, nuclear localization and coactivator interaction. Histone deacetylation neutralizes these effects. Histone deacetylation removes acetyl groups and thereby promotes the association of silencers [21].

1.2.6 Transcriptional Repression

Maintenance and *de novo* DNA methylation are associated with the silencing of gene expression through direct and indirect mechanisms. DNA-binding proteins can directly target DNMTs to promoter regions, introducing hypermethylation and repressing transcription. For example, the PML-RAR fusion protein induces gene hypermethylation and silencing by recruiting DNMT1 and DNMT3a to target the *RARβ2* promoter [9].

Indirect mechanisms involve the binding of methyl-CpG-binding proteins to methylated promoters. Methyl-CpG-binding proteins can compete with or displace other DNA-binding proteins and repress transcription of methylated promoters. A methyl-CpG-binding protein can repress transcription through the recruitment of histone-modifying complexes to methylated cytosines, which induces the formation of compact chromatin and renders the locus less accessible to necessary transcription factors [6] (Figure 1.1). Methyl-CpG-binding protein MeCP2 and methyl-CpG-binding domain proteins MBD2 and MBD3 contain histone deacetylases HDAC1 and HDAC2 and other transcriptional co-repressors. MBD3 also resides in a complex with Mi2, a chromatin remodelling protein. Therefore, methyl-CpG-binding proteins serve as a bridge between the two major epigenetic mechanisms, DNA methylation and histone modification [17].

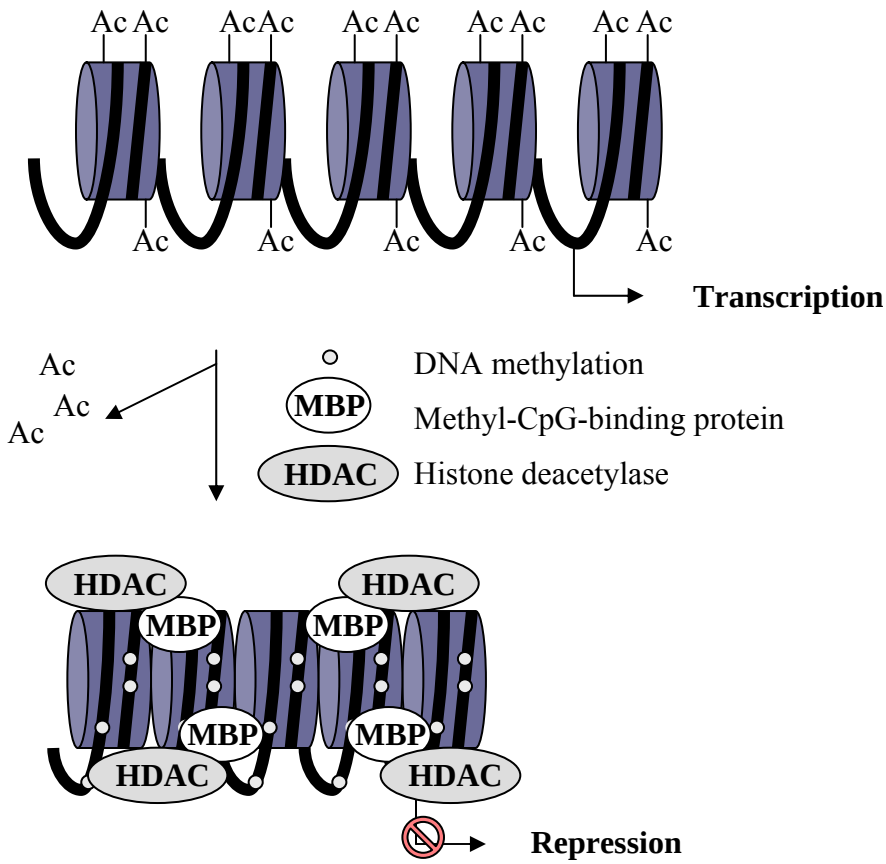

Figure 1.1 Schematic of epigenetic gene silencing.

The promoters of most actively transcribed genes may be occupied by both activating and repressing protein complexes. Gene expression levels are then determined by cellular signals influencing the amount of these complexes and post-translational modifications of proteins in these complexes [8].

The possibility that DNA methylation patterns might be controlled by the methylation status of histones emerged from genetic studies. A gene of *Arabidopsis,* Kryptonite, is required for the maintenance of CpG methylation at silenced reporter constructs and also encodes a histone methyltransferase [20]. It is possible to replace the acetyl group at H3 Lys9 with one to three methyl groups. This was shown to be essential

for DNA methylation in fungus, suggesting that histone methylation may provide a signal for DNA methylation [7]. Also, mutations in a putative histone methyltransferase result in loss of CpG methylation, suggesting that histone methylation precedes DNA methylation [18].

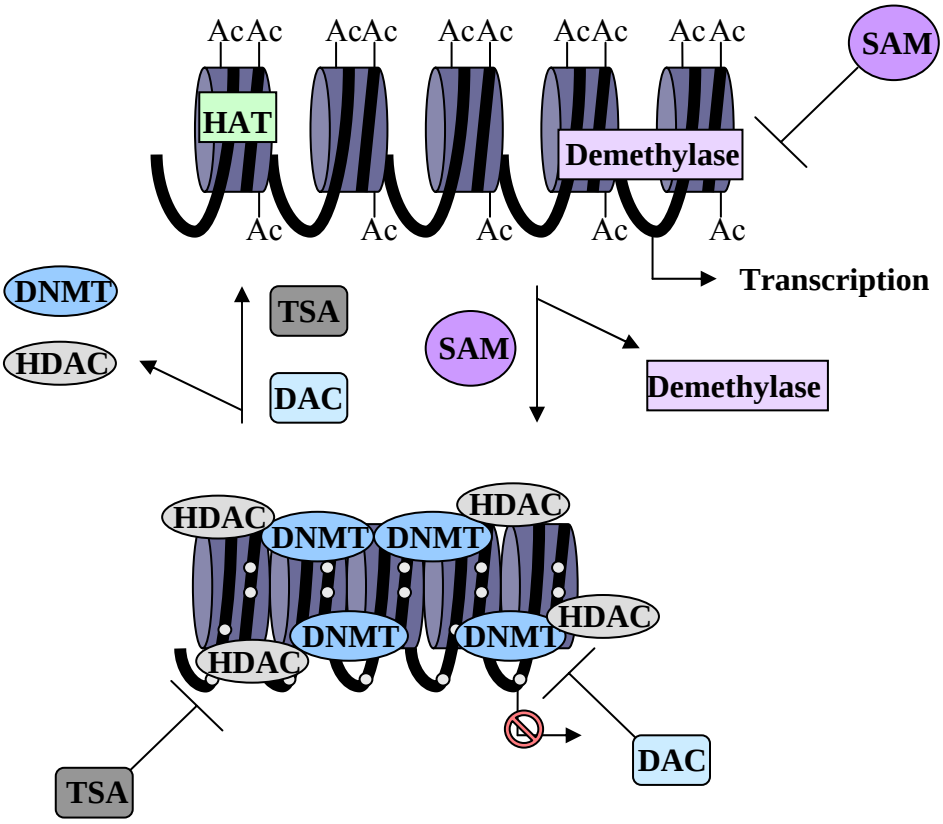

Figure 1.2 Schematic of DNMT, HDAC and DNA demethylase inhibition.

Evidence shows that pre-treatment of cells with DNMT inhibiting agents allows greater reactivation of silenced genes by treatment with histone deacetylase inhibitors (Figure 1.2). These results indicate that DNA methylation works in concert with methyl-CpG-binding proteins and histone deacetylases in epigenetic transcriptional silencing of genes [8]. Since DNA methyl-CpG-binding domain proteins and DNMT1 recruit HDACs to methylated promoters and MBD-containing corepressors associate with

methylated CpG islands, it has been suggested that histone acetylation is secondary to DNA methylation [18].

1.2.7 DNA Methylation and Tumourigenesis

Point mutations, deletions and insertions are present throughout the genome of a neoplastic cell. However, a malignant cell has also acquired a different epigenotype [5]. Epigenetically mediated loss of gene function precedes and appears to be essential for several genetic events that drive tumour progression [8].

A normal cell has to acquire several novel capabilities in order to become a *bona fide* cancer cell: limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, sustained angiogenesis and tissue invasion and metastasis [16]. A cancer cell acquires its unique characteristics through a stepwise accumulation of heritable changes in the content of proto-oncogenes, tumour suppressor genes, metastasis-promoting genes and metastasis suppressor genes. While gain, loss and mutation of genetic information commonly contribute to tumourigenesis, epigenetic mechanisms play an equally important role. Methylation of CpG islands in promoter regions of tumour suppressor genes lead to transcriptional silencing via histone deacetylation and chromatin remodelling, representing a tumourigenic event functionally equivalent to genetic changes like mutation and deletion. The two alleles of a tumour suppressor gene may be inactivated by any combination of genetic and epigenetic events [13].

The process of CpG hypermethylation is probably progressive, consisting of several steps of deregulated methylation. Cancer methylation may spread from normal

methylation centers surrounding the methylation-free CpG island. Certain CpG dinucleotides may become methylated as a result of basal methylation, creating a focus for further methylation [5]. Normally unmethylated CpG islands may become methylated in cancer cells [18]. This may occur if hypermethylation confers a selective advantage for the survival of that particular cancer cell [5]. DNA hypermethylation events may cause the inactivation of genes involved in the cellular response to chemotherapy, inactivation of 'caretaker' genes such as those involved in DNA repair and protecting cells from carcinogenic agents [13].

Loss of cell cycle control resulting in unrestrained cell proliferation is a classic event in tumourigenesis. Increased cell proliferation may required for epigenetic changes in cancer cells, since CpG islands are not remethylated in non-dividing cells suggesting that *de novo* CpG island methylation occurs only in dividing cells. Overexpression of DNMTs in normal cells can produce aberrant *de novo* methylation of CpG islands and promote cellular transformation. DNMT mRNA levels are regulated during the cell cycle. Improper DNMT expression during the cell cycle can contribute to methylation alterations in cancer cells. Hypermethylated CpG islands leave molecular footprints from which the event of epigenetic progression can be reconstructed during tumourigenesis [18].

Many cancer-related genes harbour dense methylation in normally unmethylated promoter CpG islands, which affects most cellular pathways with many consequences [8] [14]. For example, genes involved in DNA repair (*MGMT*), drug resistance, cell cycle regulation (*p16INK4a*, *p15INK4b*, *p14ARF*), growth, differentiation, apoptosis (*DAPK*), signalling, hormonal regulation (*RARβ2*), angiogenesis, cell adherence (*CDH1*),

metastasis and invasion, are associated with methylation-regulated gene silencing in different tumour types [15] [12] [5].

The *DAPK* gene was isolated as a positive mediator of interferon-γ-induced apoptosis. It also has a role in the activation of a p19ARF/p53 cell cycle checkpoint. The loss of *DAPK* expression or methylation of its associated CpG island may characterize highly invasive or metastatic tumours. Therefore, methylation or loss of expression could be associated with a metastatic phenotype [15].

Hypermethylation of the cell-cycle inhibitor gene, *p16INK4a*, enables cancer cells to escape senescence and begin to proliferate [14]. p53 is inactivated through methylation-mediated silencing of the tumour suppressor gene *p14ARF*, which normally inhibits MDM2, an oncogenic protein that induces p53 degradation. *p73*, a *p53* homologue, is shown to be hypermethylated in leukemia [14]. DNA methylation affects repair pathways by silencing DNA mismatch repair genes, hypermethylation of mitotic checkpoint genes and preventing repair of DNA double-strand breaks [14].

In cancer, promoter hypermethylation is often associated with wide spread loss of methylation throughout the genome and modest increases in expression of all three DNA methyltransferases [16]. DNA from breast carcinomas is generally hypomethylated. Global hypomethylation contributes to carcinogenesis through chromosomal instability, reactivation of transposable elements and loss of imprinting [14] [13].

It appears that some genes are predisposed to CpG island hypermethylation. Genes with the highest incidence of dense promoter hypermethylation in colon cancer appear predisposed to this change by aging. Promoter CpG islands of these genes are

slightly methylated in the normal colon of young individuals. The density of methylation increases with age and is highest in cancer [8].

Epigenetic alterations are among the most common molecular alterations in human neoplasia, resulting in a revision of Knudson's two-hit hypothesis. In addition to the traditional two possibilities: loss of heterozygosity or homozygous deletion, a third possibility can disable tumour suppressor genes: epigenetic silencing by DNA methylation and histone deacetylation within promoter regions [16].

1.3 Metastasis Suppressor Genes

Metastasis suppressor genes are defined as genes responsible for suppressing metastasis without affecting primary tumour growth [4]. Restoration of metastatic suppressor gene expression would yield cells that are still tumourigenic but are no longer metastatic. On the other hand, tumour suppressor genes suppress primary tumour growth and, by default, suppress metastasis. Metastasis genes can be identified through comparison of cell lines different in metastatic potential. Differential display, subtractive hybridization and microarray technologies are all methods of measuring differential gene expression. Identification of metastasis suppressors is much less complicated than of metastasis-promoting genes, since many genes are required for the progression of metastasis but the expression of only a single novel gene may suppress this progression [4].

There are several examples whereby the expression of metastasis suppressor genes is lost, not through conventional mutations, but via alternative epigenetic mechanisms for loss-of-function [4]. Prostatin is a serine protease that decreases

invasiveness *in vitro*. Both prostatin mRNA and protein are detected in normal breast cells, poorly invasive and nonmetastatic breast carcinoma cell lines but are not detected in highly invasive or metastatic breast carcinoma cells. Demethylation coupled with histone deacetylase inhibition mediated the reactivation of prostatin gene expression in highly invasive, metastatic cell lines. Induced expression resulted in a 50% reduction of *in vitro* invasiveness of these cells [16].

At least eight metastasis suppressor genes have been identified that show involvement in breast cancer progression [4]. The first of these is E-cadherin, which is a calcium-dependent mediator of cell-cell interactions [4]. Invasion and metastasis involves changes in the physical coupling of cells to their microenvironment and activation of extracellular processes. Epithelial cells maintain contact with their neighbours through adherens junctions. Cadherins traverse the membrane, associating with cadherins on adjacent cells [16]. Exogenous E-cadherin expression, via transfection, decreases motility and invasiveness. High E-cadherin levels inhibit shedding of tumour cells from the primary tumour and thus, E-cadherin is considered a metastasis suppressor [3]. The E-cadherin gene (*CDH1*) is located on chromosome 16q22.1, a region associated with loss of heterozygosity in cancer. However, gene loss may not solely be responsible for *CDH1* loss-of-function. Differential methylation patterns have been identified in the CpG islands of the *CDH1* promoter regions. In one study, promoter methylation was not evident in normal breast epithelium but was evident in some breast carcinomas. Hypomethylation of the *CDH1* promoter correlates with increased gene expression [4]. Direct involvement of hypermethylation in *CDH1* gene suppression was supported by the observation that its expression can be reactivated by DAC treatment [8].

Tissue inhibitors of metalloproteinases (TIMPs) inhibit the activity of matrix proteinases by forming strong noncovalent complexes. Decreased matrix proteinase activity results in less invasive cells and thus, inhibits metastasis [23]. *TIMP-2* promoter hypermethylation was detected in several leukemia and lymphoma cell lines and is associated with transcriptional repression. Treatment with the demethylating agent DAC resulted in *TIMP-2* upregulation in these cell lines [24].

Nm23 was the first novel metastasis gene discovered. The human gene, *NME1,* maps to chromosome 17q21 [4]. *NME1* is a *bona fide* metastasis suppressor gene in human breast carcinoma since transfection of metastatic MDA-MD-435 cells with *NME1* suppressed metastasis [3]. Expression of *NME1* was down-regulated in late-stage, metastatic breast, endometrial, ovarian, melanoma and colon cancer. *NME1* promoter hypomethylation was associated with increased expression, suggesting epigenetic mechanisms may regulate this gene [4]. The *nm23* gene family exhibits metastasis suppressor activity in breast cancer *in vivo*. Two CpG islands are present in the *nm23-HI* promoter. Bisulfite sequencing of these CpG islands in a panel of cell lines and in 20 infiltrating ductal carcinomas revealed that one island exhibited infrequent differential methylation. Treatment with DAC increased the *nm23-HI* expression in 5 of 11 human breast carcinoma cell lines *in vitro*, including all three metastatically competent cell lines. Increased *nm23-HI* expression was accompanied by a reduction in motility *in vitro*, with minimal effect on proliferation [16].

Maspin is a member of the serpin family of serine protease inhibitors and is located on chromosome 18q21.3-q23. In one study, six of seven mammary carcinoma cells lines that did not express maspin exhibited aberrant methylation of the maspin

promoter. Maspin expression can be restored in these cell lines by treatment with the hypomethylating agent, DAC [25].

Kai1 encodes an adhesion molecule that maps to chromosome 11p11.2, a region commonly associated with breast cancer progression [2]. Transfection of *Kai1* into MDA-MB-435 cells suppressed metastasis from the mammary fat pad [3]. *Kai1* expression inversely correlates with aggressive behaviour in breast cell lines. Survival of patients with *Kai1*-negative tumours is significantly lower than of those with *Kai1*-positive tumours. As with *NME1* and maspin, the *Kai1* promoter exhibits aberrant methylation patterns [26].

KiSS1 is located on chromosome 1q32. Studies have shown an inverse correlation of *KiSS1* expression with melanoma metastatic potential [4]. *KiSS1* expression is lost as melanoma cells convert from radial to vertical growth phase (benign to malignant transformation). Transfection of *KiSS1* into MDA-MD-435, which has no endogenous *KiSS1* expression, resulted in suppression of metastasis from the mammary fat pad of athymic mice [3].

Introduction of a portion of chromosome 17 significantly suppresses the metastatic ability of rat prostate AT6.1 cancer cells. *MKK4* was identified in this region. Transfection of a *MKK4/SEK1* expression construct into AT6.1 significantly suppressed metastasis without affecting primary tumour growth [3]. Studies on *KiSS1* and *MKK4* revealed that tumour cells defective for these two genes are able to complete every step of the metastatic cascade except growth at the secondary site, implying that these genes are metastasis suppressors [4].

The breast cancer metastasis suppressor 1 (*BRMS1*) gene is located on chromosome 11q13.1-13.2, a region commonly altered in late-stage breast carcinomas [2]. Transfection of *BRMS1* into MDA-MB-435 and MDA-MB-231 breast carcinoma cell lines suppressed metastasis without affecting tumourigenicity [3]. The BRMS1 protein is located in the nucleus, contains a glutamate-rich domain, an imperfect leucine zipper and coiled-coil domains, suggesting that it may be part of a transcription complex [4]. BRMS1 suppresses metastasis of multiple human and murine cancer cells without inhibiting tumourigenicity. It was found to interact with retinoblastoma binding protein 1 and seven members of the HDAC complex using yeast two-hybrid and co-immunoprecipitation techniques. BRMS1 was shown to be a component of an HDAC complex, contributing to transcriptional repression [27].

1.4 Relevance

While genetic mutations confer a fixed irreversible state of gene inactivation, epigenetic events do not interfere with the information content of the affected genes and are potentially reversible. Epigenetic silencing can be alleviated by two mechanisms: inhibition of DNA methylation and inhibition of histone deacetylation [13]. However, drugs used to inhibit DNMTs can also cause global hypomethylation. Lower doses of DAC combined with inhibitors of histone deacetylases such as TSA, depsipeptide, suberoylanilide hydroxamic acid and sodium butyrate may reactivate epigenetically silenced genes [14]. TSA was one of the first HDAC inhibitors identified as an anti-proliferative agent, and although it has never progressed as a clinical candidate, it has been a valuable tool in validating HDAC enzymes as potential anti-cancer targets [10].

A promising clinical scenario for the use of epigenetic therapy is acute promyelocytic leukemia (APL), which is caused by transcriptional disruption induced by the PML-RARα translocation. Inhibitors of histone deacetylases, inhibitors of DNA methylation and differentiating factors have achieved success in APL patients [14]. Inhibitors of class I/II HDACs and DNMTs are currently in clinical trials for cancer treatment. Research on these enzymes may improve the therapeutic potential of deacetylase and DNMT inhibitors in 'transcription therapy' of human diseases, including cancer [21].

CpG islands are mostly unmethylated in normal tissues but are methylated to varying degrees in human cancers, including breast cancer [16]. DNA methylation serves as a more useful tumour biomarker compared to other DNA alterations because there are usually no interindividual variations in the methylation pattern of a particular gene. The use of DNA methylation as a marker allows the establishment of highly sensitive and universally applicable assays via methylation-specific PCR. A novel microarray technique, differential methylation hybridization (DMH), allows for global analysis of DNA methylation in cancer. In ovarian cancer, this approach has revealed tumour groups with distinct methylation patterns that respond differently to chemotherapy. The use of expressed CpG island sequence tags (ECISTs) has further refined DMH. GC-rich regions of ECISTs are used to screen for methylated CpG sites in cancer cells and exon-containing fragments of ECISTs are used to measure levels of gene expression [18]. Tumour-specific DNA is easily obtained from sputum of lung cancer patients, urine from prostate cancer patients and serum of cancer patients to facilitate DMH screening [13].

Promoter hypermethylation of CpG islands in tumour suppressor genes occurs early in tumourigenesis, implicating the possibility of early detection in cancer via DMH screening [14]. Promoter hypermethylation is frequently associated with *BRCA1* silencing in non-inherited breast and ovarian carcinomas [8]. Therefore, a DNA-based approach to screening for breast cancer may complement the current mammography-based approach [16].

Genes that are inactivated by promoter hypermethylation can be used as prognostic factors. Methylation-associated silencing affects many genes in most cellular pathways. Hypermethylation of *DAPK* and *p16INK4a* have been linked with poor prognosis in colorectal cancer. Other possible aberrantly methylated genes awaiting analysis for their relation to metastatic or angiogenic activity in primary tumours include those encoding E-cadherin, H-cadherin and thrombospondin 1 [14].

Products of genes that are silenced by DNA methylation can be used as biomarkers of response to therapy. The lack of effectiveness of antisteroidal drugs is a consequence of methylation-mediated silencing of their respective cellular receptors. Premalignant lesions become insensitive to retinoids because of epigenetic silencing of genes that are crucial to the retinoid response, particularly the retinoic acid receptor. The DNA repair gene *MGMT*, encoding protein O6-methylguanine-DNA methyltransferase, undergoes transcriptional repression as a result of methylation-associated silencing. MGMT is responsible for repairing the addition of alkyl groups to guanine. Tumours that lost MGMT due to hypermethylation would be more sensitive to the action of alkylating agents since their DNA lesions could not be repaired in the cancer cell [5]. Although silencing of this gene alone is a poor prognostic factor because patients acquire more

mutations, hypermethylation of this enzyme's promoter was indicative of a good response to chemotherapy, greater overall survival and longer time in remission [14].

Hypermethylation of CpG islands occurs in conjunction with the action of methyl-CpG-binding proteins, histone hypoacetylation and histone methylation, which all contribute to formation of a closed chromatin state and transcriptional silencing. Several pharmaceutical and biotechnology companies are using novel approaches such as antisense constructs, ribozymes and RNA interference to target these elements of the methylation machinery. Some companies are investigating the use of gene-therapy-like strategies to reactivate specific methylated genes [14].

Chemoresistance is a major hindrance of effective chemotherapy. There are several cellular and molecular mechanisms resulting in chemoresistance. The expression of genes conferring chemoresistance can be reversibly turned off by DNA methylation. Chemotherapy destroys cancer cells by inducing apoptosis, or programmed cell death. Deregulation of genes involved in the activation or execution of apoptosis may serve as a mechanism for chemoresistance. For example, loss of caspase-8 expression, an apoptosis-related protein, resulted in resistance to cytotoxic drugs like doxorubicin and cisplatin. Treatment of cells containing caspase-8 promoter hypermethylation with a demethylating agent led to re-expression of caspase-8 and restored chemosensitivity [13].

1.5 Introduction to *MS-1*

1.5.1 R3230AC Model System

A rat mammary adenocarcinoma cell line was used to isolate two subpopulations differing in metastatic phenotype in order to investigate properties unique to these populations. The poorly metastasizing R3230AC rat mammary adenocarcinoma was cyclically enriched for metastasizing cells to derive the highly metastatic LN_4 subpopulation [28]. Briefly, female Fischer 344 rats were injected with 10^6 R3230AC cells in the hind footpad, which is drained by the popliteal lymph node. These cells metastasize initially through the lymphatic system making this a good model for many human tumours, such as breast cancer, which metastasize initially through the lymphatics. Infrequent lymph node metastases (10% of injected animals showed evidence of metastases) were excised, the tumour cells grown in culture and subsequently reinjected into another series of rats. The proportion of animals bearing lymph node metastases progressively increased with sequential enrichments. After three (LN_3) or four (LN_4) enrichments, the frequency of lymphatic metastasis increased to 100% of injected animals [28].

An increased percentage of cells binding high levels of the lectin soybean agglutinin (SBA) was observed with each enrichment cycle, and showed a strong correlation with the metastatic potential of these cell populations [28]. The SBA binding component on the surface of these cells was found to be a neutral glycosphingolipid, identified as isoglobotetraosylceramide (iGb_4Cer) [29] [30]. An R3230AC-LG_3 cell line, enriched for lung colony formation, also showed enrichment for iGb_4Cer expression [30].

Depletion of the subpopulation of cells containing this cell surface marker within the R3230AC-LN_4 cell line by antibody-dependent complement-mediated cytolysis using the anti-iGb_4Cer monoclonal antibody 3E9 resulted in a cell population (CAb) with a substantially decreased metastatic potential without significantly altering the primary tumour growth rate [29]. Similarly, depletion of iGb_4Cer expressing cells within the R3230AC-LG_3 cell line with antibody-dependent complement-mediated cytolysis significantly reduced lung colony formation [30]. Blocking the iGb_4Cer on the surface of LG_3 cells with the monoclonal antibody 3E9 or its Fab fragments greatly decreased lung colony formation, suggesting it may play a direct role in the metastatic process [30].

1.5.2 *MS-1* Identification and Differential Expression

The LN_4 and CAb subpopulations of our model system have many similar characteristics, such as tumourigenicity, but allow for the identification of genes that are differentially expressed and that may be involved in generating the metastatic phenotype. The production of iGb_4Cer in metastatic cells was found to be associated with the loss of the sialyltransferase enzyme G_{M3} synthase, which competes for a common intermediate in iGb_4Cer biosynthesis [31]. G_{M3} synthase was expressed in CAb cells but not in the iGb_4Cer-producing LN_4 cell line. During attempts to isolate the G_{M3} synthase cDNA, genes differentially expressed between the CAb and LN_4 cells were isolated. A unique 1 kb sequence was isolated that hybridized to a 2.8 kb mRNA in the CAb subpopulation but was undetectable in metastatic LN_4 cells (Figure 1.3). This mRNA was distinct from the reported rat G_{M3} synthase cDNA [32]. This differentially expressed novel gene was termed *MS-1* [33] [34]. The relationship between metastatic phenotype and expression of

rat *MS-1* (*rMS-1*) was also seen in a Dunning rat prostate adenocarcinoma system (Figure 1.3). The poorly metastatic AT1 subpopulation showed high levels of *rMS-1* while there was no detectable *rMS-1* expression in malignant MAT-LyLu cells [33].

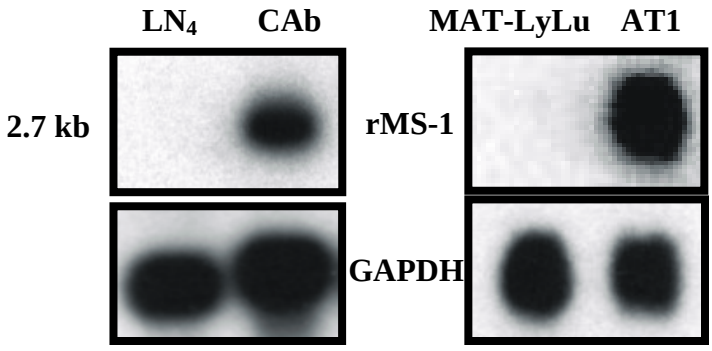

Figure 1.3 Northern analysis demonstrating the differential expression of *rMS-1*.
A total of 10 μg of total RNA was loaded in each lane, transferred and probed with a 565 bp rMS-1 specific cDNA probe or 452 bp GAPD rat specific cDNA probe. (n>2)

The observation that *MS-1* expression varied in a number of rat cell lines led to cloning and characterization of a human homologue of *rMS-1*. Northern analysis was used to screen human breast tumour cell lines for *MS-1* expression (Figure 1.4). Hs578Bst, Hs578T and WiDr cells were positive for *MS-1* expression, MDA-MB-231 cells were weakly positive for *MS-1* expression, while MCF-7, MDA-MB-435, T-47D, HEY, SW480 and SW620 cells were negative [34]. The information provided by ATCC indicates that MDA-MB-231, MDA-MB-435, MCF-7 and T-47D were cell lines derived from pleural effusion samples from female patients with advanced breast cancer. The SW480 cell line was established from a primary adenocarcinoma of the colon and the SW620 cell line was established from a lymph node metastasis taken from the same patient one year later. The WiDr cell line was established from a colon adenocarcinoma.

The malignant ovarian epithelial cell line, HEY, was established from a human ovarian carcinoma. Hs578T cells were recovered from a primary tumour of a breast cancer patient and the Hs578Bst cell line was established from normal tissue peripheral to this tumour. The majority of these results support our previous observation that *MS-1* expression is limited to cells of low metastatic potential.

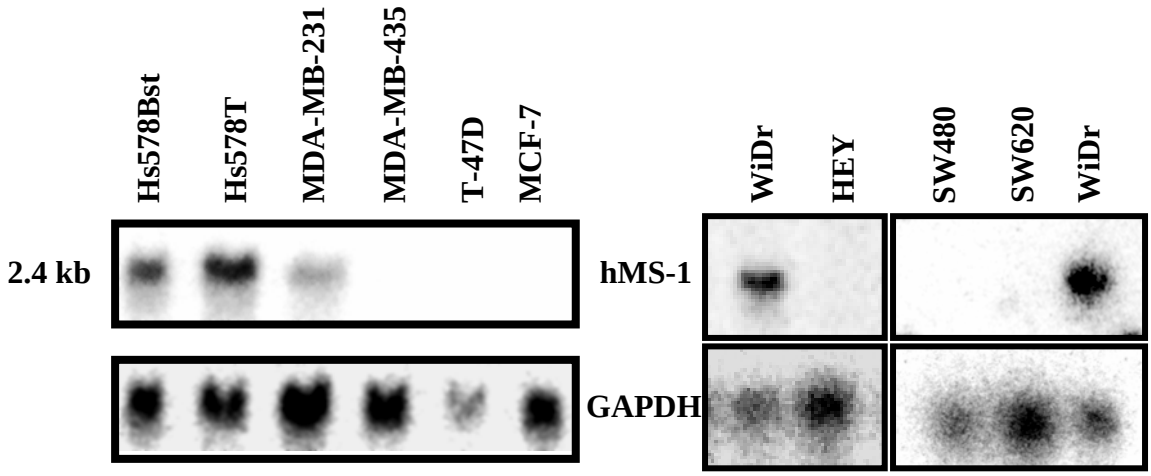

Figure 1.4 Northern analysis demonstrating the differential expression of *hMS-1*.
A total of 15µg total RNA was loaded in each lane, transferred and probed with a 558 bp hMS-1 cDNA specific probe or 452 bp GAPD human specific cDNA probe. (n>2)

1.5.3 Old Astrocyte Specifically Induced Substance (OASIS)

The murine homologue of MS-1, OASIS (Old Astrocyte Specifically Induced Substance), was identified and characterized [35]. It was specifically induced in long-term cultured mouse astrocytes, an *in vitro* model of gliosis. Gliosis, the injury response of astrocytes in the central nervous system, is characterized by the phenotypic modification, proliferation and migration of reactive astrocytes [36]. *OASIS* expression

was induced in gliotic tissues following cryo-injury of the cerebral cortex or spinal cord injury, suggesting it may play a role in gliotic events [35] [37].

OASIS was strongly expressed in embryonic tissues including salivary gland, tooth germs, bone and cartilage [35] [37]. *OASIS* showed transient upregulation in the mouse brain during the two weeks postnatal and weaker expression in the adult, suggesting it to be a developmentally regulated gene [35]. Other suggested roles include involvement in osteogenesis, as *OASIS* expression overlapped several osteogenesis markers. Expression patterns of *OASIS* throughout development of bone and cartilage were similar to expression of the gene encoding X-box binding protein, *XBP1*, another basic leucine zipper (bZIP) transcription factor [37].

It was shown by Nikaido et al. that the OASIS protein could specifically bind the cAMP response element (CRE) consensus sequence, consistent with other CREB family members [38]. A transcriptional activation domain was identified at the amino-terminus of OASIS [37] [39]. Fusion proteins with the Gal4 DNA binding domain and OASIS were able to activate transcription of luciferase reporter constructs. The minimum region of CREB3L1 required for this transcriptional activation was identified between amino acids 1 and 60 [39].

1.5.4 Role of MS-1

The evolutionary conservation of MS-1 illustrated by sequence homology between the human, rat and mouse genomes implies a functional requirement. The widespread expression of *MS-1* in tissues of all three species, including embryonic tissues, suggests that it is a normally expressed factor that may be involved in

development. Expression profiles of *OASIS* suggest roles in gliosis, salivary gland development and osteogenesis.

The MS-1 protein contains a transactivation domain, basic region, leucine zipper and transmembrane domain. Immunofluorescence analysis determined the subcellular localization of MS-1 to be within the cytosol in the vicinity of Rhodamine B-stained endoplasmic reticulum (ER). Truncation of the carboxy-terminus, which contains the transmembrane domain, altered localization to the nucleus in addition to the cytosol [34]. MS-1 is a novel CREB/ATF family transcription factor that seems to be involved in signal transduction from the ER to the nucleus. Studies indicate MS-1 is cleaved by regulated intramembrane proteolysis (RIP), producing the truncated, active MS-1 transcription factor [34]. RIP is a process that allows cells to respond quickly to physiological crises by activating pre-made transcription factors [40]. MS-1 undergoes RIP following treatment with chemical inducers of ER stress [34].

Studies on OASIS reveal that the protein is cleaved in response to ER stress and that mutations at site-1 and site-2 protease sites prevents this cleavage. Also, OASIS mRNA is induced in astrocytes following treatment with various ER stressors. OASIS has been characterized as an ER-stress transducer for the unfolded protein response (UPR). Endogenous OASIS binds to CRE and ER response element (ERSE) sequences within the BiP promoter, inducing its expression, in response to ER stress. BiP is a UPR target gene that functions as a cytoprotective protein in stress cells. It was proposed that OASIS could protect cells from ER stress. Studies showed that *OASIS*-transfected cells had a higher resistance to ER stress-induced cell death and that knockdown of *OASIS* expression caused cells to undergo apoptosis [40].

The inverse relationship between the metastatic phenotype and *MS-1* expression presents the possibility that the MS-1 protein may have a role in suppressing metastasis. As primary tumours grow in size, the cancer cells experience hypoxia, nutrient starvation and acidosis, which hinders cell proliferation and can result in cell death [41]. Cells may adapt to this ischemic environment by producing pro-angiogenic factors that initiate the formation of new blood vessels to the tumour, and allow the tumour cells to enter the bloodstream and metastasize. However, conditions within the tumour microenvironment also result in ER stress and gene expression changes through the UPR signalling pathway [41]. ER stress results in RIP cleavage of MS-1, which was shown to induce BiP expression and protect cells from ER stress-induced apoptosis. Therefore, it is possible that MS-1 protects primary tumour cells from undergoing apoptosis, even under ischemic conditions, eliminating both the requirement for angiogenesis and the opportunity for metastasis. In the absence of MS-1 there would be no induction of BiP and thus, no protection from ER stress-induced apoptosis. Tumour cells lacking *MS-1* expression would require angiogenesis for survival. It has been shown that following nutrient deprivation, hypoxia and ER stress, pro-angiogenic factors are induced in metastatic breast cancer cell lines MCF-7 and T-47D, which do not express MS-1 [41].

If MS-1 is shown to be involved in suppressing metastasis, it may play future roles as a marker for diagnosis and in the treatment of metastatic breast cancer.

1.6 Objectives

The study of epigenetic gene regulation has presented a relatively novel model for understanding the control of gene expression in the progression of various diseases,

including cancer metastasis. A number of genes appear to inhibit the metastatic process. Such genes are commonly identified on the basis that they are lost or down regulated during the progression of metastasis. The discovery of a novel gene, *MS-1*, whose expression appears limited to cells of low metastatic potential, led to the suggestion that this gene may play a role in metastasis. The presence of a CpG island in the 5' region of *MS-1* promoted an investigation of epigenetic silencing of *MS-1* in highly metastatic cells. The purpose of the following research was to investigate the mechanism by which *MS-1* expression is down regulated in cells of high metastatic potential. This research identified different methylation profiles of the *MS-1* CpG island in cells of different metastatic potential. It also uncovered possible mechanisms by which this methylation profile may be altered via reversal of epigenetic silencing mechanisms. This evidence presents a novel application for demethylating agents, which are currently in clinical trials, and provides reasoning to further research on epigenetic therapy as a possibility in cancer treatment.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

LN_4.D6 is a clone of the highly metastatic LN_4 subpopulation of the rat mammary adenocarcinoma R3230AC, selected by cyclic enrichment of increased metastatic ability [28]. These cells were found to specifically express high levels of the neutral glycolipid, isoglobotetraosylceramide. CAb.D5 is a clone selected from the poorly metastatic subpopulation (CAb), which was selected from the highly metastatic LN_4 subpopulation by depletion of cells expressing this glycolipid marker by treatment with complement and antibody against this glycolipid surface marker [29].

Both AT1 and MAT-LyLu sublines were derived from the original spontaneous dorsal prostatic adenocarcinoma tumour R3327 initially discovered in 1961 by W.F. Dunning in a 22-month-old inbred Copenhagen male rat [42]. The AT1 subline was serially passaged and rarely produced distant metastases [43]. MAT-LyLu is the metastatic subline derived from successive *in vivo* passages of the AT1 tumour [44]. MAT-LyLu readily formed metastases in the lymph nodes and the lungs. Both cell lines were obtained from the European Collection of Cell Cultures.

Hs578T cells were derived from a human breast carcinoma. The Hs578Bst cell line was established from normal tissue peripheral to this tumour [45]. MDA-MB-231, MDA-MB-435, T-47D, and MCF-7 cell lines were isolated from the pleural effusions of patients with breast carcinoma [46] [47] [48]. All human breast cancer cells were obtained from the American Type Culture Collection (ATCC) except MDA-MB-435 cells, which were kindly supplied by Dr. J. Price (MD Anderson Cancer Center, Texas). The SW480 cell line was established from a primary adenocarcinoma of the colon and the SW620 cell line was established from a lymph node metastasis taken from the same patient one year later. The WiDr cell line was established from a colon adenocarcinoma. SW480, SW620 and WiDr cell lines were kindly supplied by Dr. K. Bonham (Saskatoon Cancer Centre). The malignant ovarian epithelial cell line, HEY, was established from a human ovarian carcinoma and kindly provided by Dr. R. Hickie (University of Saskatchewan).

2.1.2 Bacterial Strains and Growth Conditions

Escherichia coli (*E. coli*) strain INVαF' [F' *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 λ-] (Invitrogen) was grown in Luria-Bertani Broth (LB, Difco laboratories) containing 1.0% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1.0% (w/v) NaCl pH 7.0. The LB ingredients were dissolved in deionized water and autoclaved for 20 min at 120ºC. For selective growth of transformed bacteria 100 μg/mL of ampicillin was added and all cultures were grown at 37°C while shaking at 300 rpm.

2.1.3 Plasmids

The plasmid pCR2.1 (Invitrogen) was used for direct cloning and sequencing of PCR purified amplifications of either *rMS-1* or *hMS-1* CpG islands.

2.1.4 Reagents and Supplies

All chemicals and enzymes used were of analytical grade or higher, and were purchased from BDH, Sigma, or VWR unless otherwise stated.

2.2 Methods

2.2.1 Tissue Culture Media and Techniques

Rat cell lines LN_4.D6, CAb.D5, MAT-LyLu and AT1 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% bovine calf serum (BCS, HyClone Laboratories, Logan, Utah, U.S.A), 100 U/mL penicillin, and 100 μg/mL streptomycin. MAT-LyLu and AT1 media was supplemented with 2 mM L-glutamine, and 250 μM dexamethasone. All media for human cells contained 10 % fetal bovine serum (FBS, Cansera), 100 U/mL penicillin, and 100 μg/mL streptomycin. Hs578Bst cells were maintained in Hybri-care medium (ATCC) with 30 ng/mL epidermal growth factor. MCF-7 cells were grown in alpha-minimal essential medium (α-MEM, Invitrogen). SW480 and SW620 cells were maintained in Dulbecco's modified eagles medium (DMEM, Invitrogen). WiDr cells were grown in minimal essential medium (MEM, Invitrogen). Hs578T, T-47D, MDA-MB-231, MDA-MB-435 and HEY cells were cultured in RPMI 1640 (Invitrogen). Hs578T and T-47D media was supplemented with

0.2 U/mL Humulin-R insulin (Lilly), as well as 2 mM L-glutamine for Hs578T cells. Cultures were maintained at 37˚C with 5% CO_2 and 100% relative humidity.

To remove all adherent cells, the existing media was removed and cells were covered in a citrate saline (pH 7.8) solution containing 0.25% (w/v) trypsin (Gibco) for 15-30 seconds before the excess solution was decanted. A thin layer of trypsin solution was left on the cells until adequate dissociation was achieved. To block the proteolytic action of the trypsin, an excess of media containing serum was added, the cells were resuspended and transferred to a 15 mL conical centrifuge tube for harvesting by centrifugation at 500 x g for 5 min. Cell pellets were resuspended in complete media, and an aliquot of the cells was seeded into a new flask containing complete media.

To appropriately store cells for later use, cells in a 175 cm^2 flask of approximately 70% confluence were harvested, as described above, and resuspended in 5 mL complete media with 10% (v/v) dimethyl sulfoxide (DMSO). Approximately 1.5 mL of cell suspension was added to 1.8 mL Cryotube™ vials (NUNC Brand Products), and the tubes were immediately placed at –70°C, and if required, transferred to liquid nitrogen the next day.

2.2.1.2 Drug Treatments

CAb, LN_4, Hs578T and MDA-MB-435 cells were grown to 60-70% confluence in 10 cm^2 plates with complete media and standard environmental conditions. Cells were treated with either 1 μM 5-aza-2'-deoxycytidine (DAC) for 96 hours and/or 1 μM trichostatin A (TSA) for 3, 6, 12 or 24 hours, and incubated at standard environmental conditions. Six day treatments with 100 μM S-adenosylmethionine (SAM) began at 20-

30% confluency. Cells were then removed from the plates, total RNA isolated and Northern analysis performed or genomic DNA isolated as described below.

2.2.2 RNA Methods

2.2.2.1 Total RNA Isolation

Total RNA was isolated using an RNeasy Kit (Qiagen). Cells were grown to approximately 80% confluency in complete media in a 15 cm^2 plate as described above. The media was removed from the plate and the cells were rinsed with phosphate-buffered saline (PBS). Cells were lysed directly in the culture dish in Buffer RLT, 4 mL for Qiagen's RNeasy Midi Kit and 400 μL for Qiagen's RNeasy Mini Kit. Total RNA was isolated according to the manufacturer's instructions for total RNA isolation from animal cells.

RNA concentrations were determined by measuring the absorbance of the RNA samples at wavelength of 260 nm using a Pharmacia Biotech Ultraspec 3000 UV/Visible spectrophotometer. Samples were stored at –70°C.

2.2.2.2 Northern Gel Electrophoresis and Transfer

A 1.2% agarose gel was prepared with 2.4 g agarose, 20 mL 10X MOPS solution (42% (w/v) 3-[N-Morpholino] propane sulfonic acid (MOPS), 0.05 M sodium acetate, pH 7.0, 0.01M Ethylenediamine tetraactetic acid (EDTA)), 170 mL DEPC water and 10.8 mL formaldehyde, and the gel was cast in a Model H5 Horizontal Gel Electrophoresis System stand (Life Technologies). The samples for electrophoresis were prepared by

combining the appropriate quantity of RNA sample with Northern blotting buffer (48 % v/v formamide, 11% v/v 10X MOPS, 17% v/v 37% formaldehyde, 12% v/v DEPC water, 7 % v/v 80% glycerol, 5% v/v 0.25% bromophenol blue solution) for a total volume of 30 μL. Each sample was heated at 65°C for 1-2 min and placed on ice for an equivalent amount of time.

The gel was run in 1X MOPS buffer for 3 hr at 70 V. To confirm the presence and stability of the RNA, the gel was washed 6 times with distilled water for 5 min each and stained with 0.33 μg/mL ethidium bromide in distilled water for 5 min. The ethidium stain was removed by 3 distilled water washes of 7-10 min each. The RNA gel was then photographed before the markers were imprinted on the gel using Pelikan Drawing ink Z (Pelikan AG, Germany).

The RNA was hydrolyzed for 15 min in 50 mM NaOH, followed by a 15 min rinse in distilled water, and neutralized in 100 mM Tris-HCl, pH 8.0 for 15 min. A final 15 min rinse with distilled water was performed before membrane transfer. Transfer to a positively charged nylon membrane (Gene Screen Plus, Perkin Elmer) was achieved using the sandwich setup [49]. The membrane and 3MM papers were soaked in 50 mM sodium phosphate, pH 6.7 prior to apparatus assembly. The transfer was carried out overnight in the presence of excess 50 mM sodium phosphate, pH 6.7.

The transfer apparatus was disassembled the following day, and the RNA size markers were impressed on the membrane for later reference. The transferred RNA was permanently associated to the membrane by UV cross-linking with 1.2×10^5 μJoules (UV Stratalinker 2400). The membrane was rinsed in 2 x SSC (300 mM NaCl, 30 mM sodium citrate) before being placed in a hybridization bag and stored at –20°C.

2.2.2.3 Northern Blot Hybridization

Probes used for hybridization were amplified from either rat or human cDNA using standard PCR conditions (described below). Table 1 outlines the primers and annealing temperatures specific for each cDNA probe. Due to high sequence homology, the same primer set can be used to amplify the GAPDH probe from either rat or human cDNA. To prepare a cDNA probe for Northern blot hybridization, 50 ng of double stranded cDNA was resuspended in TE (10 mM Tris HCl, pH 8.0, 1 mM EDTA) buffer to a final volume of 45 μL. The DNA was denatured by heating it for 5 min at 95°C, and snap cooled on ice for 5 min. The cDNA was added to the reaction tube (Amersham Biosciences Rediprime^{TM} II Random Prime Labelling System) with 50 μCi α [^{32}P]-dCTP, and mixed. The reaction was incubated at 37°C for 10 min.

The Northern membrane, as previously described, was thawed and rinsed in 0.5% SDS solution for 5 min. Prehybridization of the membrane was performed by incubating in 20-30 mL Express-hyb solution (Clontech) for a minimum of 30 min at 68°C.

The labelling reaction was stopped by adding 5 μL of 0.2 M EDTA to the reaction. The dscDNA radiolabeled probe was denatured in a boiling water bath for 5 min, snap cooled on ice for 5 min, and added to the hybridization tube containing 20-30 mL of Express-Hyb solution. The hybridization reaction was performed at 68°C for 17-22 hr. All hybridizations and subsequent washes were performed in a HYBAID Midi Oven (BIO/CAN Scientific).

Membranes were washed 3 times in wash solution #1 [2X SSC, 0.05% sodium dodecyl sulfate (SDS)] for 5-10 min each at 68°C. Each membrane was then washed 2 times in wash solution #2 (0.1X SSC, 0.1% SDS) for 20 min each at 50°C. Upon

completion, the membranes were exposed to an Imaging Screen K (Kodak) for use in a Molecular Imager FX phosphor imager (Bio-Rad). The expression of *MS-1* was standardized to the expression of a control, *GAPDH*, by densitometric analysis using Quantity One version 4.3.1 software.

2.2.2.4 Northern Blot Stripping

In order to probe a previously hybridized membrane, the cDNA probe was first removed by stripping the membrane. Membranes were soaked in approximately 1 L of boiling stripping solution (0.1% SDS, 0.1X SSC) for 20-30 min followed by 5 min in 2X SSC at 60°C. Membranes were exposed to autoradiographic film or phosphor imager screen for 2-18 hr to confirm successful removal of the previous cDNA radiolabelled probe. Once adequate stripping was achieved, all membranes were stored at –20°C.

2.2.3 DNA Methods

2.2.3.1 Genomic DNA Isolation

A QIAamp DNA Mini Kit (Qiagen) was used to extract genomic DNA from cells. A 175 cm^2 flask of each cell line of interest was grown to confluence. The cells were harvested as previously described and washed once with PBS. They were resuspended in 200 µL PBS and 20 µL of both RNase A and proteinase K was added. The genomic DNA was extracted following the manufacturer's instructions.

The DNA concentrations were determined by measuring absorbance at 260 nm using a Pharmacia Biotech Ultraspec 3000 UV/Visible spectrophotometer.

2.2.3.2 Sodium Bisulfite Modification

An EZ DNA Methylation Kit™ (Zymo Research) was used to sodium bisulfite modify isolated genomic DNA, following manufacturer's instructions. In brief, after genomic DNA isolation and purification, 2 µg of genomic DNA were treated with sodium bisulfite for 16 h. After purification, a 2-µl aliquot was used as a template for PCR using ambiguous primers specific for neither methylated nor unmethylated DNA.

2.2.3.3 PCR and Vector Construction

Different primer sets were designed to amplify either the *rMS-1* or *hMS-1* CpG island from a genomic DNA template using polymerase chain reaction (PCR) (see Table 2). Generally, 2 µL of genomic DNA was mixed in a 50 µL reaction containing 200 nM of each primer, 0.8 mM dNTPs, 1X PCR buffer (Qiagen), and 2.5 units of either Taq DNA Polymerase (Qiagen) for untreated templates or HotStarTaq DNA Polymerase (Qiagen) for sodium bisulfite modified templates in thin-walled PCR tubes. The contents were mixed thoroughly and placed into a thermocycler (Perkin-Elmer 2400). Reactions containing untreated templates were denatured for 5 min at 96°C, followed by 35 cycles with a denaturation period of 1 min at 98°C, 30 seconds for annealing (See Table 1 for annealing temperatures), and a 72°C extension interval for 2 min. Cycles were followed by a 7 min hold at 72°C and a final hold at 4ºC. Reactions containing sodium bisulfite modified templates were denatured for 15 min at 96ºC, followed by the profile described above. Nested PCR was performed using 2 µL of the primary PCR product as a template.

To verify that the fragment of interest had been specifically amplified, the product was mixed with DNA running buffer (3.7 % (w/v) EDTA, 1% (w/v) SDS, 0.1% (w/v)

bromophenol blue, 4% (v/v) glycerol), then separated on a 1% TAE (24.2 % w/v Tris, 5.7 % v/v glacial acetic acid, 0.05 M EDTA, pH 8.0) agarose gel along with a DNA marker (generally Fermentas 100 bp DNA step ladder or 1 kb marker). The gel was stained with ethidium bromide to facilitate visualization of the DNA. All DNA gels were run in 1X TAE buffer for 30-50 min at 90 V. Agarose gels were photographed using a UV transilluminator/Gel documentation system (Bio-Rad).

Table 2.1 PCR primers and annealing temperatures.

Primer Name (annealing temp ˚C)	Resulting Product (bp)	Primer Sequence (5' to 3')
SAC-223 SAC-224 (60)	1º *rMS-1* CpG58 in untreated DNA (1345)	TGGGGATTGGTGTCTTGTTAGTTC GGTTTACTTCAGGGTTTAGTGCC
SAC-225 SAC-226 (59)	Nested *rMS-1* CpG58 in untreated DNA (980)	GAAGCCAGAGACCAATGTG GTTTCCAGGATAGTTTTGCC
SAC-265 SAC-266 (51)	1º *rMS-1* CpG58 in modified DNA (1263)	TGGTGTATTTGATAGAGGTAAGGGG CRAAAACCCCCRACCCCTCCTTATC
SAC-267 SAC-257 (48)	Nested *rMS-1* CpG58 in modified DNA (995)	CCTTGTGCTTTGTTCTGGTGCTGGGC TGCCAGGTGAGGGTCAGGTAGTCC
SAC-131 SAC-302 (62)	1º *hMS-1* CpG51 in untreated DNA (1202)	GGAGACGCAGAGACAGAGGAGAG TAGGGAGAGGGAGAAAGTCAGC
SAC-134 SAC-303 (63)	Nested *hMS-1* CpG51 in untreated DNA (946)	GAGGTGGAGTCGGCTGAATGC ATCACCCTATGTTGCCCTGACC
SAC-304 SAC-305 (50)	1º *hMS-1* CpG51 in modified DNA (960)	AGTTGTGTTTTAGGAGGAGTAGG TTACCCTAACCRCCTCTTCC

SAC-306 SAC-307 (50)	Nested *hMS-1* CpG51 in modified DNA (856)	GTYGGTTGAATGTTTAYGGTG TCCCTAAAACTACCAAAATAACAC
SAC-60 SAC-68 (59)	*rMS-1* cDNA probe (565)	CTTGGGAGACCTGAATGAGTCG TGCTGGGGGGTGTTGGAGGCATC
SAC-84 SAC-85 (63)	*hMS-1* cDNA probe (558)	GCACTTTCCTGAGCACCTGGACCACTTTA TGGGAGCCGTCGCTGTCACTGC
SAC-140 SAC-141 (58)	*GAPDH* cDNA probe (452)	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA
SAC-323	pCR2.1 Sequencing (forward)	GTAAAACGACGGCCAG
SAC-324	pCR2.1 Sequencing (reverse)	CAGGAAACAGCTATGAC

For future ligation into the pCR2.1 vector (Invitrogen), 2.5 units of Taq polymerase (Qiagen) was added to the reaction tube which was incubated at 37°C for 20 min and 72°C for 20 min. This resulted in the addition of dATP to the 3' end of the PCR products for direct ligation into pCR2.1, which has a single 5' thymidine overhang. The fragment was ligated into pCR2.1 in a 10 µL reaction containing 1X ligation buffer (TA Cloning Kit, Invitrogen), ~50 ng pCR2.1 vector, ~10 ng fresh PCR product and 4 Weiss units of Quickligase (Invitrogen). The contents were incubated at room temperature for 10 min and stored at -20ºC.

2.2.3.4 Bacterial Cell Transformation

For transformations, an aliquot of frozen INVaF' competent cells was thawed on ice. The ligation reaction (3 µL) was added to the cells and placed on ice for 30 min followed by a 30 sec heat shock at 42°C. The reaction vial was placed on ice for 2 min

before 250 µL of pre-warmed SOC (2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$-$6H_2O$, 20 mM glucose) medium was added. The cells were shaken at 37°C for 1 hr at 225 rpm in a gyratory shaker.

To ensure that isolated colonies would be formed, 100 µL and 150 µL of transformed competent cells were plated on 100 mm diameter plates of LB agar containing 100 µg/mL ampicillin and 40 µL of 20 mg/mL X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) from Invitrogen. The plates were incubated at 37°C overnight. Cells transformed with a successfully ligated pCR2.1 construct formed white colonies, while those that retained the uninterrupted *lacZα* gene fragment formed blue colonies. Several white colonies were streaked on additional LB agar plates containing 100 µg/mL ampicillin for further analysis.

2.2.3.5 Plasmid DNA Isolation

A 2-5 mL LB broth (+100 µg/mL amp) culture was inoculated with a wire loop of bacterial transformant and incubated overnight in a shaker at 300 rpm and 37°C. The following day, 1.5-3.0 mL of bacterial culture was centrifuged at 9600 x g for 30-60 sec. The supernatant was aspirated off and the pellet was resuspended in QIA mini-prep-kit buffer P1 (Qiagen). Plasmid DNA was isolated according to the manufacturer's instructions.

For storage of bacterial stocks for later use, 0.75 mL of bacterial culture was combined with 0.75 mL of glycerol, mixed by inversion and stored at –70°C.

To determine if the isolated plasmid DNA contained the insert of interest, it was digested with the restriction enzyme, *Eco*RI. In a 0.5 mL microfuge tube, 4 µL of the

mini-prep DNA was mixed with 5-10 U of the appropriate restriction enzyme, and the compatible quantity of 10 X One-Phor-All Plus (100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate, 500 mM potassium acetate) (Amersham Pharmacia) buffer in a final volume of 10 µL. The digestion reaction was incubated at 37°C for a minimum of 1 hr, after which the entire reaction volume was mixed with DNA running buffer and electrophoresed on a 1% TAE agarose gel accompanied by a DNA marker.

2.2.3.6 DNA Sequencing

All DNA sequencing was performed at the National Research Council (NRC) Plant Biotechnology Institute (PBI) DNA Technologies Unit using an automated ABI Prism sequencing apparatus.

2.2.3.7 Combined Bisulfite Restriction Analysis (COBRA)

Methylation profiles were determined using bisulfite-PCR followed by restriction digestion. Ambiguous primers were used to amplify the CpG island in sodium bisulfite modified DNA. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The amplified fragments were digested with restriction enzymes that digest DNA only if the CpG sites in their recognition sequences are methylated: *Hpy*CH_4IV or *Bst*UI (New England Biolabs). Digestion with an enzyme that digests DNA only if sodium bisulfite modification of the DNA is incomplete was used as a control: *Cvi*AII (New England Biolabs). Universally methylated human DNA (Chemicon International) or rat DNA, methylated *in vitro* by CpG methylase (*Sss*I; New England Biolabs) was used as a positive control. In a 0.5 mL microfuge tube, 500-700 ng DNA was mixed with 2.5-5

U of the appropriate enzyme and 5 μL of the compatible 10X enzyme buffer in a final reaction volume of 50 μL. NEBuffer 1 (10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.0) was used with *Hpy*CH_4IV, NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) was used with *Bst*UI, and NEBuffer 4 (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9) was used with *Cvi*AII. Digestions were carried out for 1 hr at 25º, 37º and 60º for *Cvi*AII, *Hpy*CH_4IV and *Bst*UI, respectively. The reaction products were separated electrophoretically on a 2% TAE agarose gel and stained with ethidium bromide. The proportion of methylated versus unmethylated product (digested versus undigested) was quantitated by densitometric analysis, determining the density of methylation. Densitometric analysis was performed using a Bio-Rad Geldoc digital analyzer equipped with Quantity One version 4.3.1 software.

3.0 RESULTS

3.1 *MS-1* Comparative Genomics

The *rMS-1* gene is located on chromosome 3q24 of the rat genome, whereas the *hMS-1* gene is located on chromosome 11p11.2 of the human genome. The *rMS-1* and *hMS-1* genes both have two CpG islands in the 5' region, one surrounding the first exon and one between the first and second exons. Due to its location in the promoter region, the upstream CpG island was investigated for involvement in epigenetic regulation. This CpG island in *rMS-1* is 644 bp in length and contains 58 CpG dinucleotides, whereas the *hMS-1* CpG island is 671 bp in length and contains 51 CpG dinucleotides. The *rMS-1* gene generates a 2672 bp mRNA transcript while the *hMS-1* gene generates a 2370 bp mRNA transcript, both consist of twelve exons. The mRNA sequence of rMS-1 shares 75% of its sequence with the mRNA of hMS-1 (Figure 3.2). The ORF of rMS-1 encodes a 520 amino acid protein, whereas the ORF of hMS-1 encodes a 519 amino acid protein.

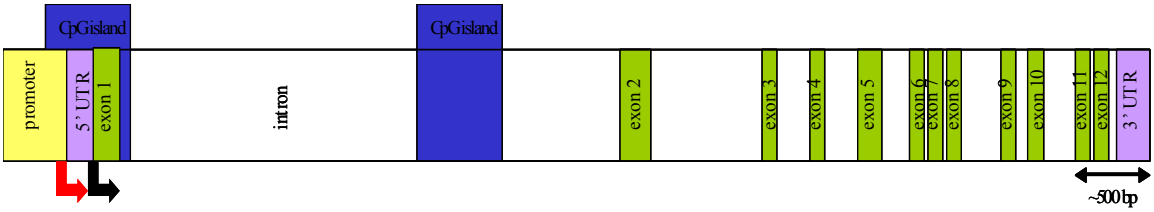

Figure 3.1 *MS-1* gene.
The promoter region is highlighted in yellow, untranslated regions are purple, exons are green, introns are white, CpG islands are highlighted in blue, the putative transcriptional start site is indicated by a red arrow and the translational start site by a black arrow.

Figure 3.2 Aligned rMS-1 and hMS-1 mRNA sequences. Regions of homology are highlighted.

3.2 Induction of *MS-1* Expression

3.2.1 *MS-1* Induction following DAC and TSA Treatment

The mechanism by which *MS-1* expression is lost in cells of high metastatic potential was unclear. Southern analyses indicate the presence of the *MS-1* gene in all rat and human cell lines investigated, suggesting that regulation mechanisms and not LOH are responsible for differential expression [34]. The presence of a CpG island surrounding the first exon of *MS-1* points to epigenetic mechanisms as possible modes of regulation. DNMT and HDAC inhibitors can be used to reverse epigenetic gene silencing. Previously obtained data suggest gene expression may be induced in LN_4 or MDA-MB-435 cells by treatment with a DNMT inhibitor and a histone deacetylase inhibitor [33] [34].

5-aza-2'-deoxycytidine (DAC) inhibits DNA methyltransferases (DNMTs), which are responsible for transferring the methylation profile of parental strands to daughter strands during DNA replication. Therefore, DAC requires 96 hours to fully deplete the DNA of methylation. Trichostatin A (TSA) is a histone deacetylase inhibitor. HDACs are responsible for removing acetyl groups from histone proteins. Acetyl groups are important for retaining a loose chromatin structure necessary for transcription.

To investigate the possible role of epigenetics in the regulation of MS-1, highly (LN_4, MDA-MB-435) and poorly (CAb, Hs578T) metastatic rat and human cell line pairs were treated with 1 μM DAC for 96 hrs followed by 1 μM TSA for 0, 3, 6, 12 or 24 hrs. Following drug treatments, total RNA was isolated from the cells and gel electrophoresis and Northern blotting was performed to detect the level of *MS-1* expression. Quantitation

using a phosphor imager revealed that, although treatment for 96h with DAC or 24h with TSA alone caused an average induction of *rMS-1* expression in LN_4 cells 4-fold and 3-fold, respectively, the greatest average induction, 12-fold, occurred when both drugs were used in conjunction (Figure 3.3). On average, *hMS-1* expression was induced 4-fold in MDA.MB.435 cells at 96h DAC plus 24h TSA (Figure 3.4). These observations suggest that both DNA methylation and histone deacetylation may be involved in epigenetically silencing the *MS-1* gene in highly metastatic LN_4 and MDA-MB-435 cells. In order to validate this observation, a more extensive examination of the methylation status of the *MS-1* CpG islands was carried out.

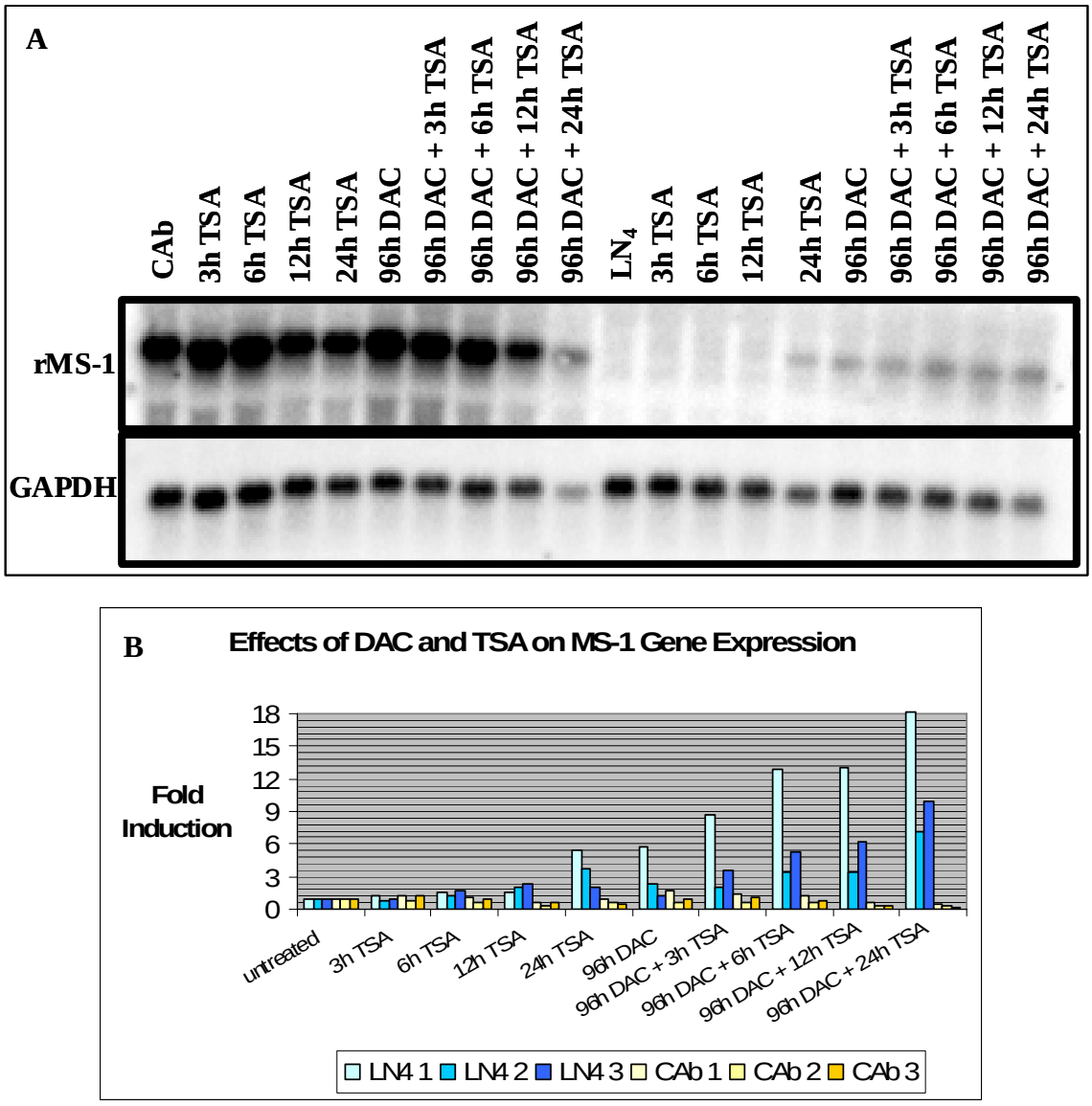

Figure 3.3 DAC and TSA induced *rMS-1* expression.
A. CAb and LN_4 cells were treated with 1 μM 5-aza-2'-deoxycytidine (DAC) and/or 1 μM Trichostatin A (TSA). A total of 10 μg of total RNA was loaded in each lane, transferred and probed with a 565 bp *rMS-1* specific cDNA probe or rat *GAPDH* specific cDNA probe. B. Graphical overview of triplicate Northern analyses. Each bar represents a single experiment (n=3).

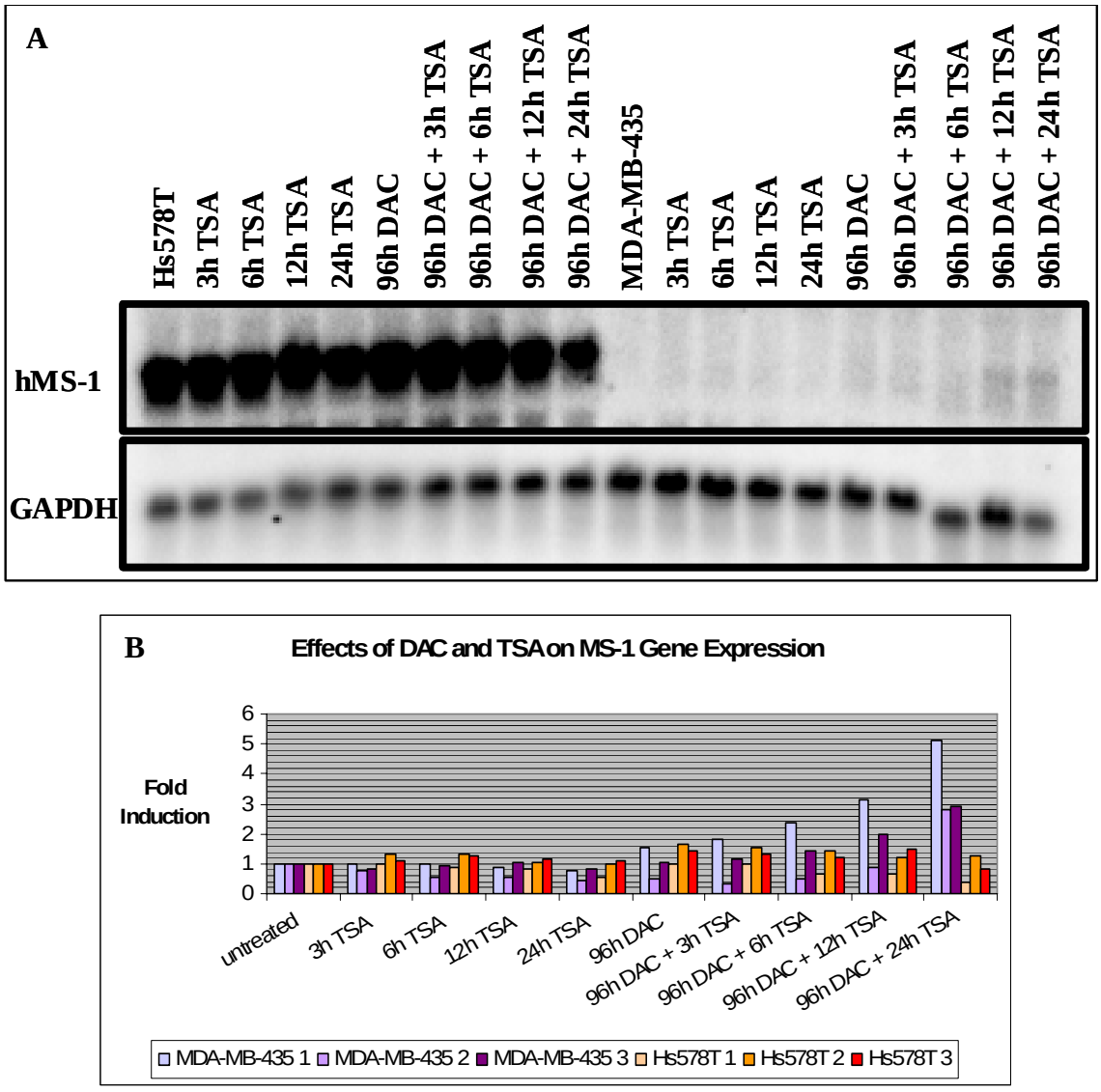

Figure 3.4 DAC and TSA induced *hMS-1* expression.
A. Hs578T and MDA-MB-435 cells were treated with 1 μM 5-aza-2'-deoxycytidine (DAC) and/or 1 μM Trichostatin A (TSA). A total of 10 μg of total RNA was loaded in each lane, transferred and probed with a 558 bp *hMS-1* cDNA specific probe or 452 bp human *GAPDH* specific cDNA probe. B. Graphical overview of triplicate Northern analyses. Each bar represents a single experiment (n=3).

3.2.2 *MS-1* Induction following SAM Treatment

Since the methylation profile of a CpG island is determined by a balance between DNMT and demethylase activity, inhibition of demethylase should increase CpG methylation and reduce expression of genes with normally unmethylated promoter regions. It has been shown that S-adenosylmethionine (SAM) actively inhibits demethylase and can reduce expression of the metastasis-promoting gene urokinase plasminogen activator (*uPA*) [1], and has been proposed as a potential anti-cancer treatment [19]. To investigate the specificity of SAM, both rat and human highly (LN_4, MDA-MB-435) and poorly (CAb, Hs578T) metastatic cell line pairs were treated for 6 days with 0.1 mM SAM, total RNA extracted and Northern analysis was performed. Quantitation using a phosphor imager revealed an induction, not reduction, of *MS-1* expression in already expressing, poorly metastatic cells. An average 3-fold induction of *rMS-1* was observed in CAb cells (Figure 3.5) and an average 2-fold induction of *hMS-1* in Hs578T human cells (Figure 3.6). These results suggest that SAM treatment may alter gene expression by mechanisms other than inhibiting demethylase actively. In order to shed light on whether the induction of *MS-1* expression is methylation-independent, a more extensive investigation of the methylation status of the CpG islands following SAM treatment was carried out.

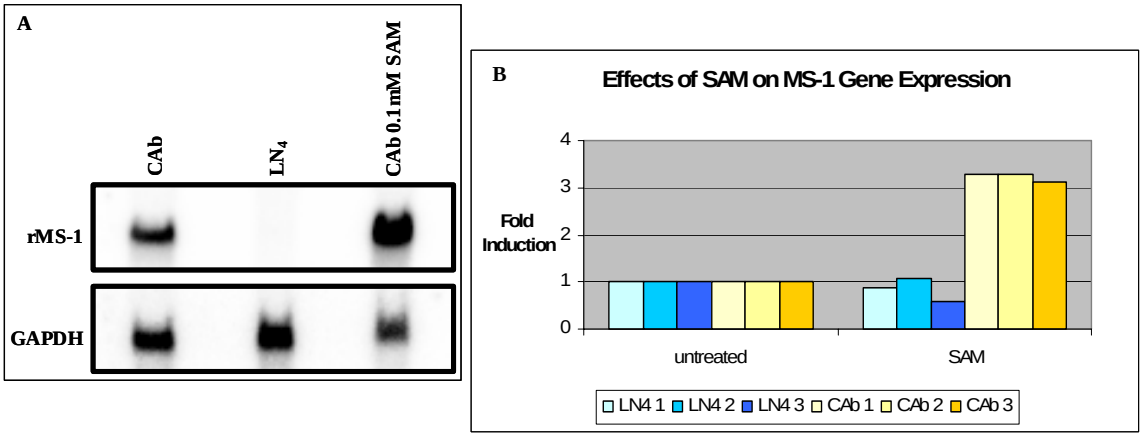

Figure 3.5 SAM induced *rMS-1* expression.
A. CAb and LN_4 cells were treated with 0.1mM S-adenosylmethionine (SAM) for 6 days. A total of 10 µg of total RNA was loaded in each lane, transferred and probed with a 565 bp rat *MS-1* specific cDNA probe or 452 bp rat *GAPDH* specific cDNA probe. B. Graphical overview of triplicate Northern analysis. Each bar represents a single experiment (n=3).

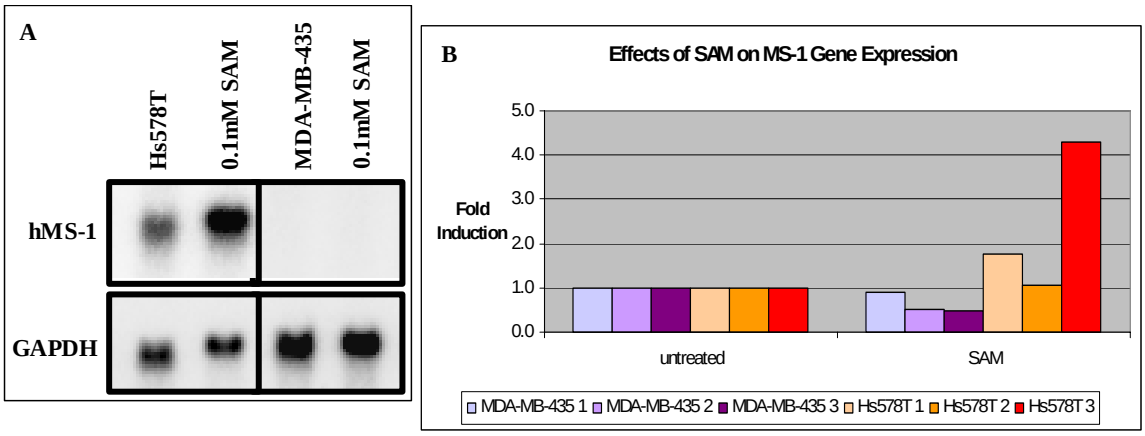

Figure 3.6 SAM induced *hMS-1* expression.
A. Hs578T and MDA-MB-435 cells were treated with 0.1mM S-adenosylmethionine (SAM) for 6 days. A total of 10 µg of total RNA was loaded in each lane, transferred and probed with a 558 bp human *MS-1* cDNA specific probe or 452 bp human *GAPDH* specific cDNA probe. B. Graphical overview of triplicate Northern analysis. Each bar represents a single experiment (n=3).

3.3 Methylation Profiles of *MS-1* CpG Islands

3.3.1 Methylation of CpG Islands in Cells that do not express *MS-1*

Two methods were utilized to investigate the methylation profiles of the CpG islands of MS-1 in various cell lines: combined bisulfite restriction analysis and bisulfite sequencing. The first method used was bisulfite sequencing. Sodium bisulfite deaminates unmethylated cytosines but does not modify methylated cytosine bases, discriminating between methylated and unmethylated cytosines of CpG islands. The outside and nested primers designed to amplify modified DNA were ambiguous, selecting for neither methylated nor unmethylated DNA. Primer sets SAC-265/266 and SAC-267/257 amplified the CpG island of *rMS-1*, while SAC-304/305 and SAC-306/307 amplified the CpG island of *hMS-1* (Table 2.1). The PCR products were purified, cloned into the vector pCR2.1, and three clones were sequenced. The number of deaminated nonCpG cytosines divided by the total number of nonCpG cytosines gave a percent of modification in each sample. The number of CpG cytosines protected from deamination by a methyl group divided by the total number of CpG dinucleotides gave a percent of methylation in each sample. The percent methylation was then multiplied by the percent modification to give a standardized percent methylation.

All samples, except those from LN_4 DNA, showed 97-100% modification, indicating that the sodium bisulfite had deaminated nearly all nonCpG cytosines and the treatment had gone to near completion. After several attempts, LN_4 DNA was, at most, 64-71% modified by sodium bisulfite, for unknown reasons. For *MS-1* expressing cell lines CAb and Hs578T, the CpG islands revealed an average standardized methylation of

2.8% and 0%, respectively. The CpG islands of the cell lines that did not express *MS-1*, LN_4 and MDA-MB-435, showed an average standardized methylation of 38.7% and 69.2%, respectively. Following treatment with DAC and TSA, the average standardized methylation fell to 19.5% and 33.1% in the CpG islands of LN_4 and MDA-MB-435, respectively. These data indicate a correlation between hypermethylation of the CpG island and lack of *MS-1* expression. Also, DNMT and HDAC inhibition resulted in a significant depletion in the methylation of these CpG islands.

Table 3.1 Triplicate bisulfite sequencing analysis of *rMS-1* and *hMS-1* CpG islands.

Sample	Percent Modification	Percent Methylation	Standard % Methylation	Average
CAb 1	99	0	0	
CAb 2	97	8.6	8.4	2.8
CAb 3	99	0	0	
LN_4 1	64	53.5	34.4	
LN_4 2	65	53.5	35.0	38.7
LN_4 3	71	65.5	46.6	
LN_4 DAC+TSA 1	100	0	0	
LN_4 DAC+TSA 2	100	29.3	29.3	19.5
LN_4 DAC+TSA 3	100	29.3	29.3	
Hs578T 1	100	0	0	
Hs578T 2	100	0	0	0
Hs578T 3	100	0	0	
MDA-MB-435 1	100	62.8	62.8	
MDA-MB-435 2	100	74.5	74.5	69.2
MDA-MB-435 3	100	70.6	70.6	
435 DAC+TSA 1	98.9	27.5	27.2	
435 DAC+TSA 2	99.5	7.8	7.8	33.1
435 DAC+TSA 3	99.5	64.7	64.4	

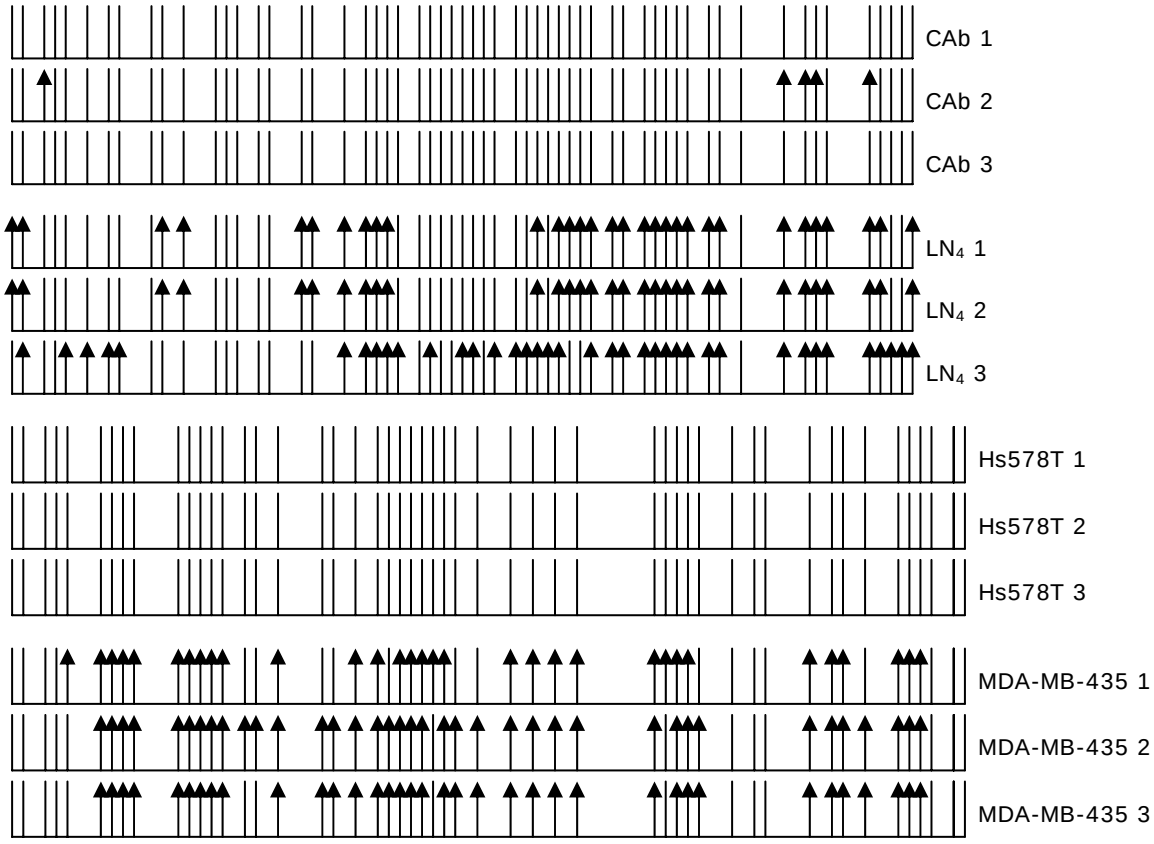

Figure 3.7 Methylation profiles of *rMS-1* CpG Island 58 and *hMS-1* CpG Island 51. Bars indicate CpG dinucleotides and black triangles indicate methylation, detected following triplicate bisulfite sequencing analysis.

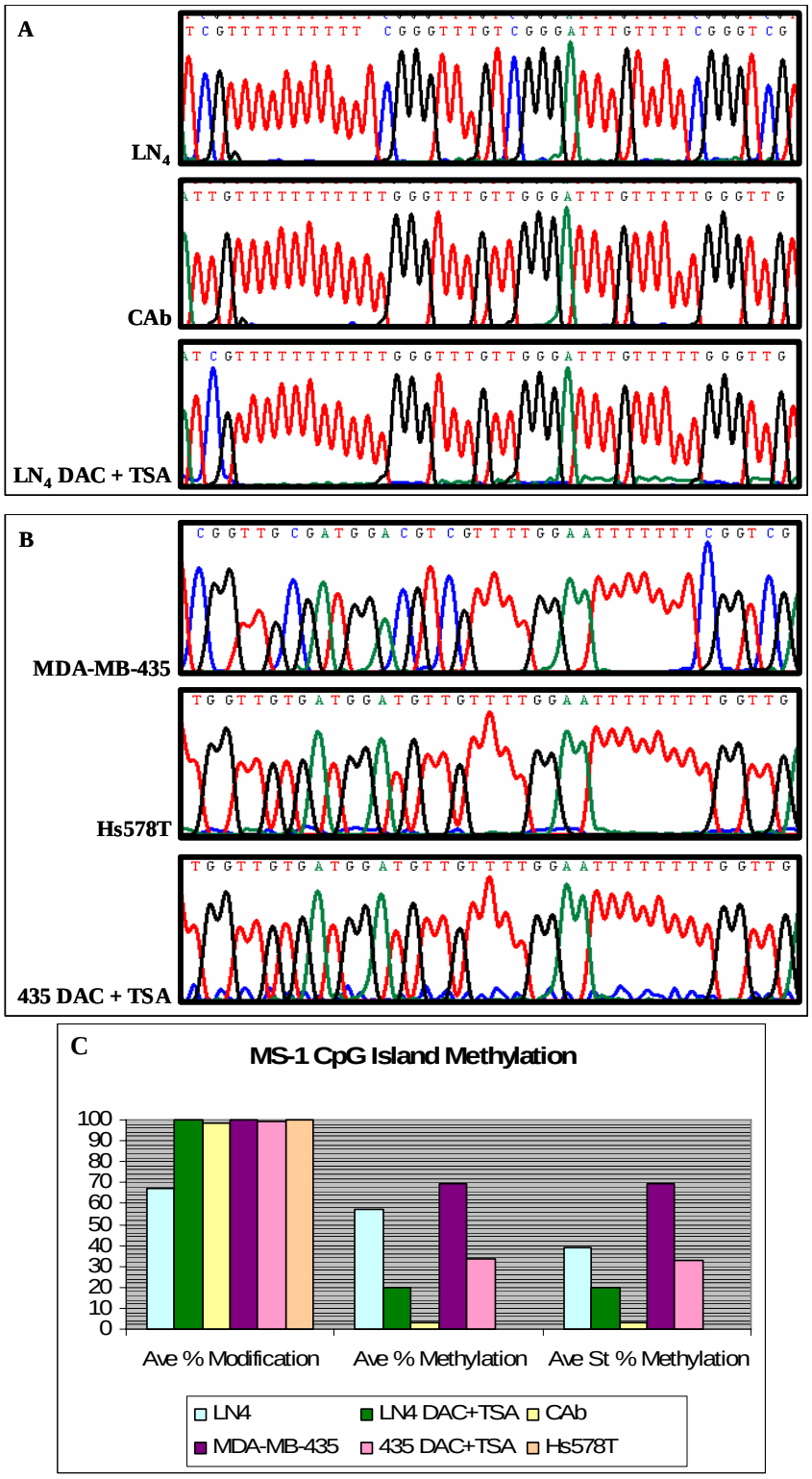

Figure 3.8 Representative sequences of the *MS-1* CpG islands.
A. *rMS-1* sequence analysis. B. *hMS-1* sequence analysis. C. Graphical overview of the average bisulfite sequencing results from three individually cloned PCR products.

3.3.2 Screening Various Cancer Cell Lines for Methylation

The second method used to investigate the methylation profiles of the *MS-1* CpG islands was combined bisulfite restriction analysis. The same primers used to amplify the *rMS-1* and *hMS-1* CpG islands for bisulfite sequencing were also used for restriction analysis (Table 2.1). Restriction digestion was performed using a control enzyme, *Cvi*AII, which cuts at CATG sites. Following bisulfite modification these sites should be eliminated, and only unmodified DNA will be digested. Therefore, the proportion of DNA undigested versus digested by this enzyme was used to calculate the percent modification for each sample. Enzymes *Hpy*CH_4IV and *Bst*UI cut at ACGT and CGCG sites, respectively, and thus digest only unmodified DNA and methylated DNA following bisulfite modification. The proportion of DNA digested versus undigested by these enzymes was quantitated by densitometric analysis in order to determine the density of methylation. The results from enzymes *Hpy*CH_4IV and *Bst*UI were combined to give an average percent methylation of the CpG islands from each sample.

COBRA was performed on both rat and human poorly and highly metastatic cancer cell lines in order to determine the CpG island methylation profiles. Of the rat cell lines, CAb and AT1 are both poorly metastatic, *MS-1* expressing cell lines, whereas LN_4 and MAT-LyLu are highly metastatic cell lines that do not express *MS-1* (Figure 1.3). *Sss*I CpG methylase treated CAb DNA was included as a positive control. Of the human cell lines, Hs578Bst, Hs578T, WiDr and MDA-MB-231 expressed *MS-1*, although MDA-MB-231 was a low expresser, whereas MDA-MB-435, MCF-7, T-47D, HEY, SW480 and SW620 did not express *MS-1* (Figure 1.4). Universally methylated human DNA (Chemicon International) was included as a positive control.

The CpG islands from *Sss*I CAb, LN_4, LN_4 DAC + TSA, LN_4 SAM and MAT-LyLu showed an average methylation of 59.3%, 58.6%, 28.7%, 33.0% and 13.5%, respectively. The CpG islands of CAb, CAb DAC + TSA, CAb SAM and AT1 contained an average methylation of 7.6%, 5.1%, 3.4% and 2.0%, respectively (Figure 3.10). The CpG islands from universally methylated human DNA, MDA-MB-435, 435 DAC + TSA, 435 SAM, MCF-7, HEY, SW480 and SW620 showed an average methylation of 92.7%, 75.1%, 16.4%, 65.8%, 41.0%, 45.3%, 15.5% and 28.8%, respectively. The CpG islands of Hs578T, Hs578T DAC + TSA, Hs578T SAM, Hs578Bst, MDA-MB-231, T-47D and WiDr all contained an average of 0.3-1.7% methylation (Figure 3.11). In all DNA samples, excluding T-47D, methylation within the CpG island corresponds to a lack of *MS-1* expression.

Following treatment with DAC and TSA, a decrease in methylation of the CpG islands in both LN_4 and MDA.MB.435 cell lines was observed. These results suggest that inhibition of DNMTs and HDACs can reduce the amount of methylation within a CpG island. However, treatment with SAM resulted in no change of methylation in the CpG island of CAb or Hs578T cells. It was predicted that SAM inhibition of demethylase would increase methylation of CpG islands and reduce expression of *MS-1*. However, from Northern analysis an increase in *MS-1* expression was observed in CAb and Hs578T cell lines. Together, these data suggest that the induction of *MS-1* expression by SAM treatment resulted from a methylation-independent mechanism.

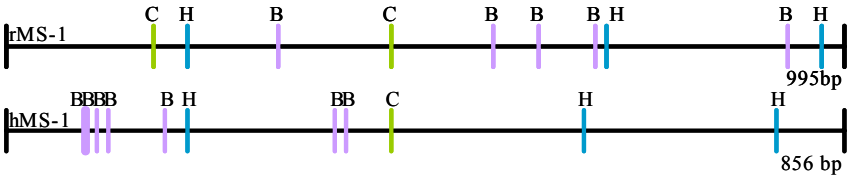

Figure 3.9 Restriction maps of *rMS-1* CpG Island 58 and *hMS-1* CpG Island 51. Restriction sites for *Cvi*AII (C), *Hpy*CH_4IV (H) and *Bst*UI (B) are indicated.

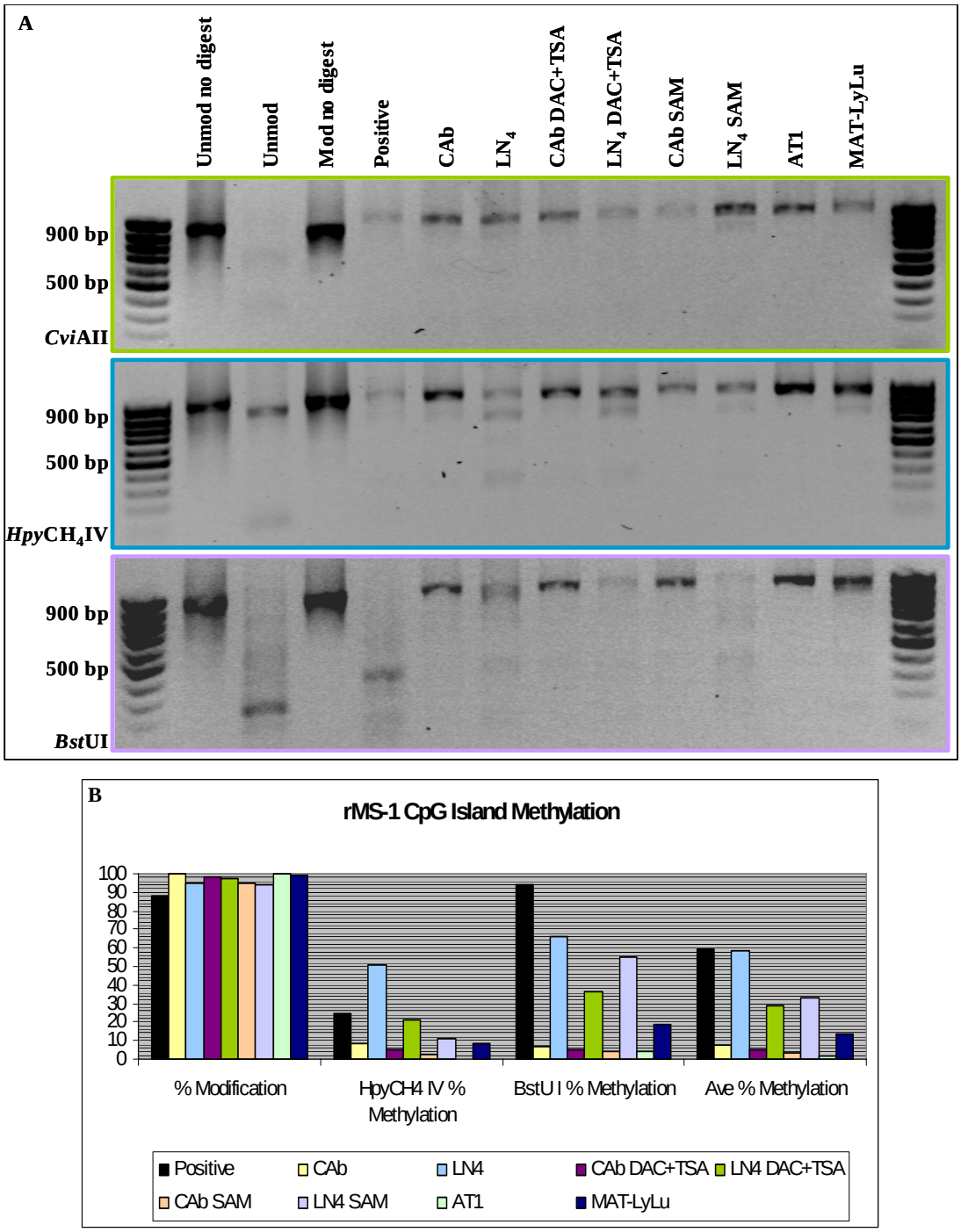

Figure 3.10 Combined Bisulfite Restriction Analysis of *rMS-1* CpG Island 58.
A. Restriction digests of 500-700 ng DNA with 2.5-5 U of each enzyme for 1 hr at the appropriate temperature. Reaction products were separated electrophoretically on a 2% TAE agarose gel and stained with ethidium bromide. Densitometric analysis was performed using a Bio-Rad Geldoc digital analyzer equipped with Quantity One version 4.3.1 software. B. Graphical overview of COBRA.

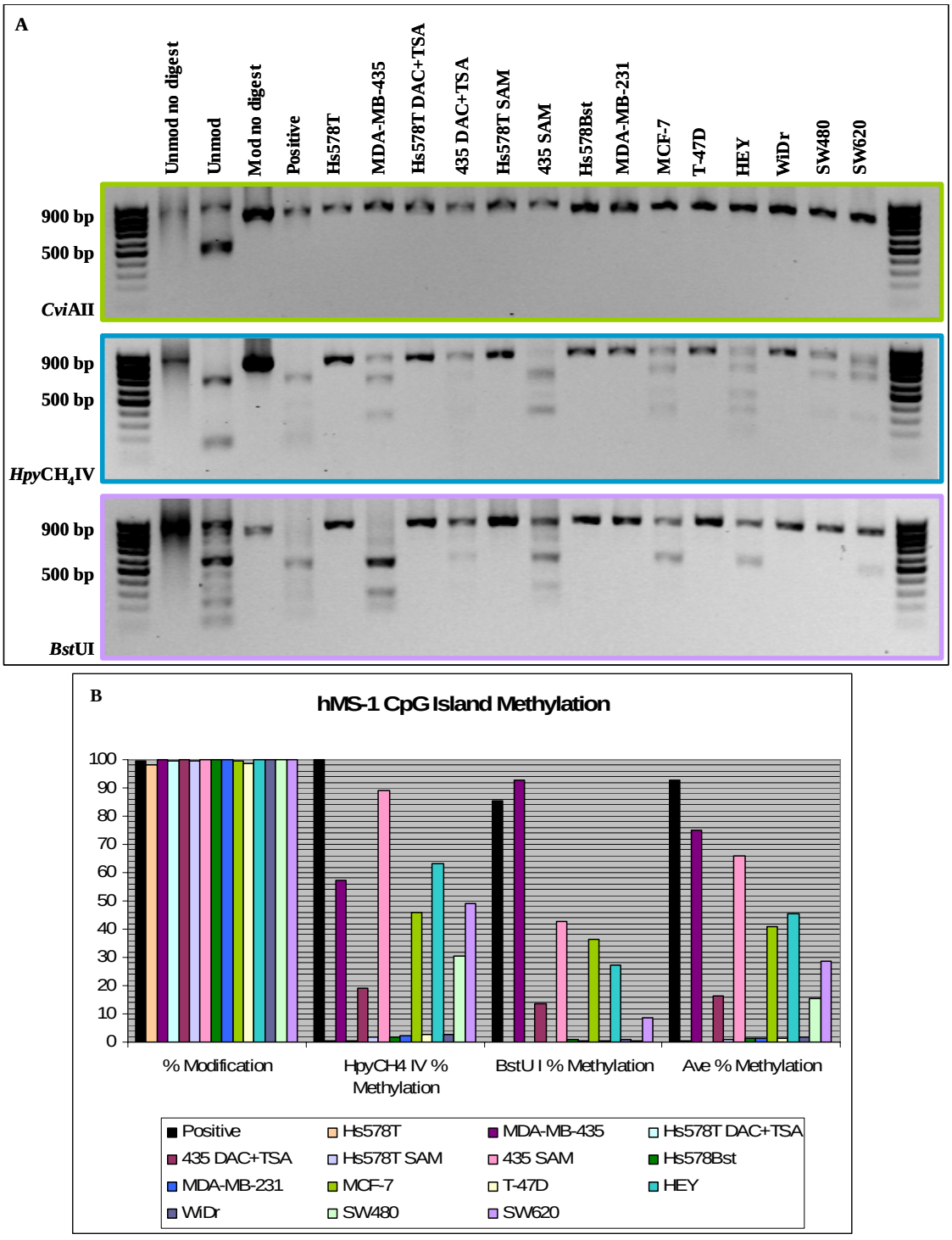

Figure 3.11 Combined Bisulfite Restriction Analysis of *hMS-1* CpG Island 51.
A. Restriction digests of 500-700 ng DNA with 2.5-5 U of each enzyme for 1 hr at the appropriate temperature. Reaction products were separated electrophoretically on a 2% TAE agarose gel and stained with ethidium bromide. Densitometric analysis was performed using a Bio-Rad Geldoc digital analyzer equipped with Quantity One version 4.3.1 software. B. Graphical overview of COBRA.

4.0 DISCUSSION

4.1 *MS-1* Expression in Cells of Different Metastatic Potential

Highly metastatic and poorly metastatic subpopulations derived from the same tumour allow for the identification of genes that are differentially expressed between the subpopulations and thus, may be involved in the metastatic phenotype. A highly metastatic subpopulation, LN_4, and poorly metastatic subpopulation, CAb were derived from the R3230AC rat mammary adenocarcinoma. A novel gene, termed *MS-1*, was found to be differentially expressed between these two cell subpopulations. Although high expression was observed in the poorly metastatic CAb subpopulation, LN_4 cells showed no expression of this gene [33].

Upon examination of other rat and human, highly and poorly metastatic cell lines, a similar pattern of expression was observed. Highly metastatic rat prostate cells, MAT-LyLu, did not express *MS-1* but the poorly metastatic counterpart, AT1, showed high expression [33]. *MS-1* expression was undetectable in human mammary adenocarcinoma cell lines T-47D, MCF-7 and MDA-MB-435, and was observed at low levels in the MDA-MB-231, which were all developed from metastatic pleural effusions. *MS-1* expression was also not observed in the human ovarian cancer cell line, HEY, highly metastatic human colon cancer cell line, SW620, or its poorly metastatic counterpart, SW480. *MS-1* expression was detected in a human normal mammary cell line,

Hs578Bst, the corresponding primary ductal carcinoma cell line, Hs578T and a poorly metastatic human colon cancer cell line, WiDr [34].

In most cell lines examined, *MS-1* expression appears to correlate with a poorly metastatic phenotype, implicating a possible role for MS-1 in suppressing metastasis. Metastasis suppressor gene *BRMS1* is located on chromosome 11q13.1-13.2, a region commonly altered in late-stage breast carcinomas [4]. Metastasis suppressor gene *K*ai1 is located on chromosome 11p11.2, a region commonly associated with breast cancer progression [4]. Introduction of human chromosome 11 into rat prostate cancer cells of high metastatic potential resulted in metastasis suppression without affecting growth rate or tumourigenicity [50]. The region between 11p11.2-13 was determined to be the minimal portion of human chromosome 11 capable of suppressing metastasis [51]. The location of the *hMS-1* gene on chromosome 11p11.2 of the human genome is consistent with the possibility that it may play a role in metastasis suppression. However, any evidence supporting the involvement of MS-1 in metastasis, if any, remains to be determined.

The mechanism by which *MS-1* expression is lost or down-regulated in cells of high metastatic potential is unknown. Although its expression was not observed in MDA-MB-435, MCF-7 and T-47D cells following Northern analysis, Southern analysis revealed that these cell lines all retained both copies of the *hMS-1* gene [34]. Therefore, LOH does not appear to be the mechanism by which *MS-1* expression was lost.

4.2 Epigenetic Regulation

A common mechanism for gene silencing is DNA methylation and chromatin remodelling. Methylated DNA, deacetylated histones, some methylated histone forms and condensed chromatin are associated with inaccessible DNA and repressed or silenced gene expression. However, unmethylated DNA, acetylated histone forms and open chromatin are associated with active or potential gene expression [6]. DNA methylation occurs at cytosine residues of CpG dinucleotides. The CpG dinucleotide has been progressively depleted from the eukaryotic genome and is found at 1/5 of the expected frequency. Remaining CpG dinucleotides outside of CpG islands have a high incidence, ~70%, of methylation [8] [13]. Small stretches in the genome (0.5 to 2kb) contain the expected frequency of CpGs (G/C content of 50% or higher and an observed CpG/expected CpG of 0.60 or higher), and are referred to as CpG islands [8] [15]. The presence of a CpG island in the 5' region of the *MS-1* gene suggests that epigenetic mechanisms may be involved in *MS-1* gene silencing.

An investigation of the methylation status of the 5' *MS-1* CpG islands was carried out to determine a role of methylation in epigenetic regulation of this gene. Bisulfite sequencing revealed that the CpG islands of *MS-1* expressing cell lines, CAb and Hs578T, contained no significant methylation. Only the CpG islands of *MS-1* in the cell lines that show no expression, LN_4 and MDA-MB-435, were significantly methylated (Table 3.1 and Figure 3.8). Combined bisulfite restriction analysis was performed to screen CpG islands from several rat and human cancer cell lines. Again, the CpG islands of *MS-1* in expressing cell lines, CAb, AT1, Hs578T, Hs578Bst, MDA-MB-231 and WiDr, were not significantly methylated. However, the CpG islands of *MS-1* in cell lines

that show no expression, LN4, MAT-LyLu, MDA-MB-435, MCF-7, HEY, SW480 and SW620, contained significant methylation (Figures 3.10 and 3.11). With the exception of T-47D, which does not express *MS-1* but whose CpG island was unmethylated, these data indicate a correlation between hypermethylation of the CpG island and lack of *MS-1* expression in various cancer cell lines.

It has been hypothesized that methylation is a third pathway, in addition to intragenic mutations and loss of chromosomal material, for complete inactivation of a gene [52]. Although Southern analysis ruled out LOH as reason for *MS-1* gene inactivation, it is possible that mutations may contribute to loss of *MS-1* gene expression. Perhaps in the T-47D cell line intragenic mutations, without promoter methylation, are responsible for *MS-1* gene inactivation.

4.3 Inhibition of DNA Methyltransferase and Histone Deacetylase Activity

Demethylation and histone deacetylase inhibition can be induced by the addition of epigenetic regulators to cells *in vitro*. The DNA methyltransferase inhibitor, DAC, and histone deacetylase inhibitor, TSA, can be used to reverse epigenetic silencing. Although treatment with DAC or TSA alone can induce expression of *rMS-1* in LN_4 cells 4-fold and 3-fold, respectively, the greatest average induction occurs when both drugs are used in conjunction, 12-fold (Figure 3.3). *hMS-1* expression is induced 4-fold in MDA-MB-435 cells following treatment with DAC plus TSA (Figure 3.4). These results indicate that DNA methylation and histone deacetylation may play individual roles in the process of epigenetic silencing but that there exists a link between the two mechanisms, allowing them to work in concert. Methyl-CpG-binding proteins can recruit histone-

modifying complexes to methylated cytosines, inducing the formation of compact chromatin and rendering the locus less accessible to necessary transcription factors [6]. Therefore, it has been proposed that methyl-CpG-binding proteins serve as a bridge between the two major epigenetic mechanisms, DNA methylation and histone modification [17].

The induction of *MS-1* expression by DAC and TSA as shown in Figures 3.4 and 3.5 supports the hypothesis that both DNA methylation and histone deacetylation may be involved in epigenetically regulating the *MS-1* gene in highly metastatic LN_4 and MDA-MB-435 cells. Examination of the methylation status of the *MS-1* CpG islands following inhibition of DNA methyltransferase and histone deacetylase activities was used to verify whether changes in methylation are responsible for the changes in expression.

Bisulfite sequencing revealed that, following treatment with DAC and TSA, the average methylation of the *MS-1* CpG islands was reduced by 50% in the highly metastatic cells, LN_4 and MDA-MB-435 (Figure 3.8). These observations validate that the induction of *MS-1* expression by DNA methyltransferase and histone deacetylase inhibition occurred alongside a significant depletion in the methylation of the *MS-1* CpG islands. The results obtained from the combined bisulfite restriction analysis confirmed the observations made from bisulfite sequencing and Northern analysis. Following treatment with DAC and TSA, a 50% or greater decrease in methylation of the CpG islands in both LN_4 and MDA-MB-435 cell lines was seen (Figures 3.10 and 3.11). Together, these results support the hypothesis that inhibition of DNA methyltransferase and histone deacetylase activity reduces the amount of methylation within the *MS-1* CpG islands, resulting in *MS-1* expression.

4.4 Inhibition of Demethylase Activity

Since the methylation profile of a CpG island may result from a balance between DNMT and demethylase activity, inhibition of demethylase may increase CpG methylation and reduce expression of genes with normally unmethylated promoter regions. This was demonstrated with the pro-metastatic gene, urokinase Plasminogen activator (*uPA*) [1]. It was predicted that SAM inhibition of demethylase would increase methylation of CpG islands and reduce expression of *MS-1* in expressing cell lines, CAb and Hs578T. However, it was demonstrated through combined bisulfite restriction analysis that treatment with SAM resulted in no significant change of methylation in the CpG island of CAb or Hs578T cells (Figures 3.10 and 3.11). Also, Northern analysis indicated an average 3-fold and 2-fold induction of *MS-1* in CAb and Hs578T cell lines, respectively (Figures 3.5 and 3.6). Together, these data suggest that the induction of *MS-1* expression by SAM treatment was caused by a methylation-independent mechanism.

The methylation-independent induction of *MS-1* by SAM, observed in CAb and Hs578T cells, contradicts the role of SAM as a demethylase inhibitor. However, other examples exist where drugs used for epigenetic therapy contradict the traditional model for epigenetic silencing. The mechanism by which HDAC inhibitors bring about changes in gene expression is poorly understood but is generally assumed to result from local changes in histone acetylation status. The observation that many common co-activators such as p300 have intrinsic HAT activity, while repressor complexes such as Sin3 are associated with HDAC activity, has strengthened this model [53]. Following this model, TSA, a known histone deacetylase inhibitor, contributes to alleviating the compact chromatin structure of transcriptionally silent genes. However, this does not fully explain

its role in regulating gene expression. It has been well characterized that treatment with TSA leads to the induction of *p21WAF1*, which mediates cell cycle arrest, and to the inhibition of c-*myc*, which mediates proliferation and differentiation. Treatment with TSA also represses expression of *c-Src*, a known oncogene, in various cell lines including T-47D, a human breast cancer cell line [53].

Histone deacetylase inhibitors are described as having anti-cancer activity, inducing cell cycle arrest, differentiation and apoptosis. These properties are presumed to result from highly selective gene expression changes. It has been shown through microarray and differential display analysis that TSA acts on only a small subset (~2%) of genes [54]. Therefore, in contrast to the idea of HDACs as master and global regulators of transcription, it appears that inhibition of HDAC leads to a fairly restricted alteration of gene expression profile which, in turn, may explain the apparent low toxicity seen in clinical trials. To date, genes which are modulated by HDAC inhibitors suggest a pleiotropic effect on key pathways involved with proliferation, apoptosis, tumour suppressors, DNA synthesis and repair, and protein turnover [10].

Although local acetylation changes can explain the induction of genes via histone deacetylase inhibitors, it is more difficult to explain the mechanism of gene repression by this treatment. Since histone acetyltransferases (HATs) are capable of acetylating non-histone proteins, it has been presumed that HDAC inhibitors can mediate the down-regulation of genes in a histone acetylation/deacetylation-independent manner [53]. To support this, studies have shown that the induction of specific serine/threonine phosphatases were required for the repression of c-*myc* by an HDAC inhibitor.

Therefore, a revised model of how HDAC inhibitors alter gene expression may include phosphorylation events as well as histone modifications [53].

Likewise, the traditional model of epigenetic silencing defines SAM as a methyl donor and inhibitor of active demethylation [1]. Studies on the pro-metastatic gene, *uPA*, shows that SAM treatment causes hypermethylation of the gene's promoter region, inhibition of the gene's expression and decreased invasiveness of normally metastatic breast cancer cells, MDA-MB-231 [1]. However, microarray analysis has shown that SAM treatment induced *Bcl-x*$_S$ expression, a pro-apoptotic factor. Just as HDAC inhibitor TSA is defined both by its role in epigenetics and by its anti-cancer properties, demethylase inhibitor SAM is defined by its role in epigenetics and by anti-cancer characteristics. There are extensive data from animal as well as some human data that suggests dietary intake of folates, which are required for the synthesis of SAM, can influence cancer progression. It was shown in animal studies that low methyl diets could induce liver cancer. Human studies suggest that low folate intake combined with high alcohol intake could result in an increased risk of colorectal cancer. There is also some evidence that folate metabolism and the resultant supply of methyl moieties might play a role in breast cancer since pre-menopausal women with the polymorphism in methylene-tetrahydrofolate reductase (MTHFR), an enzyme required for the synthesis of methionine, a precursor of SAM, had a three-fold increased breast cancer risk. SAM was shown to be chemoprotective for liver cancer in chemically induced rat liver cancer [1].

Also, just as TSA utilizes both acetylation/deacetylation-dependent and acetylation/deacetylation-independent mechanisms to regulate gene expression, SAM can alter gene expression via methylation-dependent and methylation-independent

mechanisms. Recent evidence suggests that in the liver, SAM has critical functions in modulating growth and apoptotic responses, some of which are independent of methylation because they can be mimicked by 5'-methylthioadenosine (MTA), which is not a methyl donor. Both SAM and MTA selectively up-regulated *Bcl-x*$_S$ expression in a time- and dose-dependent fashion, with the effective dose for MTA lower than that for SAM, suggesting that the effect of SAM may be mediated in part by MTA [55]. Although MTA is a metabolite of SAM, it can also be converted back to SAM via methionine. However, blocking this conversion did not prevent the induction of *Bcl-x*$_S$ expression by MTA, supporting the notion that MTA exerts its action directly. Inhibitors of histone deacetylase and DNA methylation also had no effect on the induction of *Bcl-x*$_S$ expression by SAM and MTA, thereby excluding their involvement [55].

Finally, just as the induction of specific serine/threonine phosphatases is required for the repression of c-*myc* by an HDAC inhibitor, inhibition of specific serine/threonine phosphatases nullifies SAM and MTA induction of *Bcl-x*$_S$ [55]. Therefore, a revised model of how demethylase inhibitors specifically alter gene expression may include phosphorylation events to induce anti-cancer factors as well as DNA modifications to repress genes involved in cancer and metastasis progression.

4.5 Conclusions

An inverse relationship exists between the expression of *MS-1* and metastatic phenotype in most cell lines studied. Data from bisulfite sequencing and combined bisulfite restriction analysis confirmed that hypermethylation of the *rMS-1* and *hMS-1* CpG islands correlates with the lack of *MS-1* expression. *MS-1* expression can be

induced in non-expressing cells of high metastatic potential following DNA methyltransferase and histone deacetylase inhibition. This induction occurred following hypomethylation of the CpG islands, confirmed by bisulfite sequencing and combined bisulfite restriction analysis. Therefore, epigenetic silencing via DNA methylation and histone deacetylation is one of the mechanisms involved in down-regulating *MS-1* expression in cells of high metastatic potential.

MS-1 expression can also be induced in expressing cell lines of low metastatic potential following treatment with a demethylase inhibitor. Data from combined bisulfite restriction analysis determined that this induction resulted from no change in the methylation status of the CpG island. Therefore, although inhibiting demethylase with S-adenosylmethionine can reduce expression of a metastasis-promoting gene in a methylation-dependent manner [1], treatment with this drug can also induce gene expression of a putative metastasis suppressor through methylation-independent mechanism.

4.6 Future Directions

The ability to induce *MS-1* expression through inhibition of DNMTs presents a possible role in breast cancer treatment. Decitabine, the commercial name for the DNA methylation inhibitor DAC, is currently in phase II clinical trials for the treatment of patients with chronic myelogenous leukemia [56]. However, the mere observation of an inverse relationship between *MS-1* expression and metastatic potential does not warrant the use of this drug to treat metastatic cancer. It is extremely important that the role of MS-1, if any, in metastasis suppression be identified. Animal studies are required to

determine if the expression of *MS-1* can prevent metastasis. It then needs to be determined if the inhibition of DNMTs and HDACs via drug treatments are able to induce *MS-1* expression in an animal model to the level required to carry out a suppressive role. The expression of *MS-1* and/or pro-angiogenic factors needs to be investigated in breast cancer cell lines of high and low metastatic potential following ER stress to evaluate if the role of MS-1 in the UPR relates to metastasis. The possible role of MS-1 as a transcription factor leads to the hypothesis that the induction of its expression may have a complex impact on many cellular functions, which need to be examined.

5.0 REFERENCES

1. Szyf, M., P. Pakneshan, and S.A. Rabbani, *DNA methylation and breast cancer.* Biochem Pharmacol, 2004. 68(6): p. 1187-97.
2. Welch, D.R.a.W., L.L, *Genetic and epigenetic regulation of human breast cancer progression and metastasis.* Endocr Relat Cancer, 1998. 5: p. 155-197.
3. Welch, D.R., P.S. Steeg, and C.W. Rinker-Schaeffer, *Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis.* Breast Cancer Res, 2000. 2(6): p. 408-16.
4. Debies, M.T. and D.R. Welch, *Genetic basis of human breast cancer metastasis.* J Mammary Gland Biol Neoplasia, 2001. 6(4): p. 441-51.
5. Esteller, M., *Profiling aberrant DNA methylation in hematologic neoplasms: a view from the tip of the iceberg.* Clin Immunol, 2003. 109(1): p. 80-8.
6. Fitzpatrick, D.R. and C.B. Wilson, *Methylation and demethylation in the regulation of genes, cells, and responses in the immune system.* Clin Immunol, 2003. 109(1): p. 37-45.
7. Felsenfeld, G. and M. Groudine, *Controlling the double helix.* Nature, 2003. 421(6921): p. 448-53.
8. Baylin, S.B. and J.G. Herman, *DNA hypermethylation in tumorigenesis: epigenetics joins genetics.* Trends Genet, 2000. 16(4): p. 168-74.
9. Pradhan, S. and P.O. Esteve, *Mammalian DNA (cytosine-5) methyltransferases and their expression.* Clin Immunol, 2003. 109(1): p. 6-16.
10. McLaughlin, F. and N.B. La Thangue, *Histone deacetylase inhibitors open new doors in cancer therapy.* Biochem Pharmacol, 2004. 68(6): p. 1139-44.
11. Claudet, C., et al., *Histone octamer instability under single molecule experiment conditions.* J Biol Chem, 2005. 280(20): p. 19958-65.
12. Claus, R. and M. Lubbert, *Epigenetic targets in hematopoietic malignancies.* Oncogene, 2003. 22(42): p. 6489-96.
13. Worm, J. and P. Guldberg, *DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy.* J Oral Pathol Med, 2002. 31(8): p. 443-9.
14. Esteller, M., *Relevance of DNA methylation in the management of cancer.* Lancet Oncol, 2003. 4(6): p. 351-8.
15. Farrell, W.E. and R.N. Clayton, *Epigenetic change in pituitary tumorigenesis.* Endocr Relat Cancer, 2003. 10(2): p. 323-30.
16. Widschwendter, M. and P.A. Jones, *DNA methylation and breast carcinogenesis.* Oncogene, 2002. 21(35): p. 5462-82.
17. Burgers, W.A., F. Fuks, and T. Kouzarides, *DNA methyltransferases get connected to chromatin.* Trends Genet, 2002. 18(6): p. 275-7.
18. Nephew, K.P. and T.H. Huang, *Epigenetic gene silencing in cancer initiation and progression.* Cancer Lett, 2003. 190(2): p. 125-33.
19. Detich, N., et al., *The methyl donor S-Adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of S-Adenosylmethionine.* J Biol Chem, 2003. 278(23): p. 20812-20.

20. Goll, M.G. and T.H. Bestor, *Histone modification and replacement in chromatin activation.* Genes Dev, 2002. 16(14): p. 1739-42.
21. Yang, X.J. and E. Seto, *Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression.* Curr Opin Genet Dev, 2003. 13(2): p. 143-53.
22. Ng, H.H. and A. Bird, *Histone deacetylases: silencers for hire.* Trends Biochem Sci, 2000. 25(3): p. 121-6.
23. Toi, M., S. Ishigaki, and T. Tominaga, *Metalloproteinases and tissue inhibitors of metalloproteinases.* Breast Cancer Res Treat, 1998. 52(1-3): p. 113-24.
24. Galm, O., et al., *Inactivation of the tissue inhibitor of metalloproteinases-2 gene by promoter hypermethylation in lymphoid malignancies.* Oncogene, 2005. 24(30): p. 4799-805.
25. Sager, R., et al., *Maspin: a tumor suppressing serpin.* Curr Top Microbiol Immunol, 1996. 213 (Pt 1): p. 51-64.
26. Jackson, P., et al., *Methylation of a CpG island within the promoter region of the KAI1 metastasis suppressor gene is not responsible for down-regulation of KAI1 expression in invasive cancers or cancer cell lines.* Cancer Lett, 2000. 157(2): p. 169-76.
27. Meehan, W.J., et al., *Breast cancer metastasis suppressor 1 (BRMS1) forms complexes with retinoblastoma-binding protein 1 (RBP1) and the mSin3 histone deacetylase complex and represses transcription.* J Biol Chem, 2004. 279(2): p. 1562-9.
28. Buckley, N.D. and S.A. Carlsen, *Involvement of soybean agglutinin binding cells in the lymphatic metastasis of the R3230AC rat mammary adenocarcinoma.* Cancer Res, 1988. 48(6): p. 1451-5.
29. Carlsen, S.A., M. Barry, and K. Newton, *The identification of a neutral glycosphingolipid antigenic marker for metastatic cells in the R3230AC rat mammary adenocarcinoma.* Clin Exp Metastasis, 1990. 8(2): p. 141-51.
30. Carlsen, S.A., et al., *Isoglobotetraosylceramide is a marker for highly metastatic cells in rat mammary adenocarcinomas.* Cancer Res, 1993. 53(12): p. 2906-11.
31. Dumonceaux, T. and S.A. Carlsen, *Isogloboside biosynthesis in metastatic R3230AC cells results from a decreased GM3 synthase activity.* Arch Biochem Biophys, 2001. 389(2): p. 187-94.
32. Ishii, A., et al., *Expression cloning and functional characterization of human cDNA for ganglioside GM3 synthase.* J Biol Chem, 1998. 273(48): p. 31652-5.
33. Calvert, B., *Identification and characterization of a novel rat gene, MS-1, expressed in poorly metastatic rat mammary and prostate adenocarcinomas*, in *Microbiology and Immunology*. 2002, University of Saskatchewan: Saskatoon.
34. Deibert, L., *Cloning and characterization of the human homologue of a novel transcription factor, MS-1, discovered in an R3230AC rat model system*, in *Microbiology and Immunology*. 2004, University of Saskatchewan: Saskatoon.
35. Honma, Y., et al., *Identification of a novel gene, OASIS, which encodes for a putative CREB/ATF family transcription factor in the long-term cultured astrocytes and gliotic tissue.* Brain Res Mol Brain Res, 1999. 69(1): p. 93-103.
36. Malhotra, S.K., T.K. Shnitka, and J. Elbrink, *Reactive astrocytes--a review.* Cytobios, 1990. 61(246-247): p. 133-60.

37. Nikaido, T., et al., *Expression of OASIS, a CREB/ATF family transcription factor, in CNS lesion and its transcriptional activity.* Brain Res Mol Brain Res, 2002. 108(1-2): p. 129-38.
38. Roesler, W.J., G.R. Vandenbark, and R.W. Hanson, *Cyclic AMP and the induction of eukaryotic gene transcription.* J Biol Chem, 1988. 263(19): p. 9063-6.
39. Omori, Y., et al., *OASIS is a transcriptional activator of CREB/ATF family with a transmembrane domain.* Biochem Biophys Res Commun, 2002. 293(1): p. 470-7.
40. Kondo, S., et al., *OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes.* Nat Cell Biol, 2005. 7(2): p. 186-94.
41. Marjon, P.L., E.V. Bobrovnikova-Marjon, and S.F. Abcouwer, *Expression of the pro-angiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 by human breast carcinomas is responsive to nutrient deprivation and endoplasmic reticulum stress.* Mol Cancer, 2004. 3: p. 4.
42. Dunning, W.F., *Prostate Cancer in the Rat.* Natl Cancer Inst Monogr, 1963. 12: p. 351-69.
43. Isaacs, J.T., et al., *Genetic instability coupled to clonal selection as a mechanism for tumor progression in the Dunning R-3327 rat prostatic adenocarcinoma system.* Cancer Res, 1982. 42(6): p. 2353-71.
44. Isaacs, J.T., G.W. Yu, and D.S. Coffey, *The characterization of a newly identified, highly metastatic variety of Dunning R 3327 rat prostatic adenocarcinoma system: the MAT LyLu tumor.* Invest Urol, 1981. 19(1): p. 20-3.
45. Hackett, A.J., et al., *Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines.* J Natl Cancer Inst, 1977. 58(6): p. 1795-806.
46. Cailleau, R., et al., *Breast tumor cell lines from pleural effusions.* J Natl Cancer Inst, 1974. 53(3): p. 661-74.
47. Cailleau, R., M. Olive, and Q.V. Cruciger, *Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization.* In Vitro, 1978. 14(11): p. 911-5.
48. Keydar, I., et al., *Detection of viral proteins in mouse mammary tumors by immunoperoxidase staining of paraffin sections.* Proc Natl Acad Sci U S A, 1978. 75(3): p. 1524-8.
49. Southern, E.M., *Detection of specific sequences among DNA fragments separated by gel electrophoresis.* J Mol Biol, 1975. 98(3): p. 503-17.
50. Ichikawa, T., et al., *Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11.* Cancer Res, 1992. 52(12): p. 3486-90.
51. Dong, J.T., et al., *KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2.* Science, 1995. 268(5212): p. 884-6.
52. Jones, P.A. and P.W. Laird, *Cancer epigenetics comes of age.* Nat Genet, 1999. 21(2): p. 163-7.
53. Kostyniuk, C.L., et al., *The ubiquitous and tissue specific promoters of the human SRC gene are repressed by inhibitors of histone deacetylases.* Oncogene, 2002. 21(41): p. 6340-7.

54. Dehm, S.M. and K. Bonham, *SRC gene expression in human cancer: the role of transcriptional activation.* Biochem Cell Biol, 2004. 82(2): p. 263-74.
55. Yang, H., et al., *S-adenosylmethionine and its metabolite induce apoptosis in HepG2 cells: Role of protein phosphatase 1 and Bcl-x(S).* Hepatology, 2004. 40(1): p. 221-31.
56. Issa, J.P., et al., *Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate.* J Clin Oncol, 2005. 23(17): p. 3948-56.